

MODULATORY FUNCTION OF SEROTONIN IN RAT NEOCORTEX:
5-HT_{2A} AND N-METHYL-D-ASPARTATE RECEPTOR INTERACTIONS

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**MODULATORY FUNCTION OF
SEROTONIN IN RAT NEOCORTEX:
5-HT_{2A} AND N-METHYL-D-ASPARTATE
RECEPTOR INTERACTIONS**

By

© Shafiqur Rahman, M. Sc.

A thesis submitted to the School of Graduate Studies
in partial fulfilment of the requirements for
the degree of Doctor of Philosophy

Faculty of Medicine
Memorial University of Newfoundland

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Newfoundland

ABSTRACT

The aim of this thesis was to study the functional interaction between serotonin (5-hydroxytryptamine; 5-HT) and N-methyl-D-aspartate (NMDA) receptors *in vitro* in a rat neocortical slice preparation. A quantitative pharmacological assay was developed using grease-gap recording to investigate this problem. The specific aims were: 1) to characterize the 5-HT receptor mediating facilitation of the NMDA response; 2) to elucidate the mechanism underlying the 5-HT facilitation 3) to examine the 5-HT facilitation in senescent rats; and 4) to compare the action of 5-HT with other agonists that activate G-protein coupled receptors that utilize phospholipase C signal transduction.

Depolarization of cortical neurons by NMDA, but not quisqualate or kainate, was facilitated by 5-HT (1-100 μ M) giving a bell shaped concentration-response curve. Methysergide, ritanserine and spiperone antagonized the 5-HT facilitation suggesting mediation by 5-HT_{2A} receptors. The facilitation was mimicked by DOI, a 5-HT_{2A} receptor agonist, but not by TFMPP, a 5-HT_{2C} and 5-HT_{1B} receptor agonist, or 8-OH-DPAT, a 5-HT_{1A} receptor agonist. The 5-HT facilitation was not mimicked by activating protein kinase C or occluded by blocking K⁺ currents.

The 5-HT_{2A} receptor exhibited both acute and long term desensitization.

Inhibition of protein kinase C reduced acute desensitization as did blocking receptor internalization with concanavalin A. Calmodulin antagonists prevented long term desensitization. 5-HT induced heterologous desensitization of the phenylephrine and carbachol facilitation which was eliminated by myo-inositol. Myo-inositol (1-10 mM) potentiated the 5-HT facilitation in a concentration dependent manner.

Thapsigargin, cyclopiazonic acid and A23187 mimicked the 5-HT facilitation with respect to the dependence on external Ca^{2+} , this facilitation was inhibited by nifedipine and eliminated by BAPTA-AM. A mixture of prazosin, scopolamine, D,L-AP3, ritanserin and tetrodotoxin reduced, but did not abolish the facilitation produced by thapsigargin, cyclopiazonic acid or A23187.

The 5-HT and DOI facilitation was absent in senescent rats. In contrast, agonists at muscarinic cholinergic receptors, α_1 -adrenoceptors and metabotropic glutamate receptors facilitated the NMDA depolarization in senescent rats. Inhibitors of protein kinase C restored the 5-HT facilitation in senescent rats. *In situ* hybridization histochemistry revealed that 5-HT_{2A} receptor mRNA was present in pyramidal neurons in both young adult and senescent rats.

The metabotropic glutamate receptor agonists quisqualate and 1S,3R-ACPD, like 5-HT, facilitated the NMDA induced depolarization. D,L-AP3 and D,L-AP4

inhibited the 1S,3R-ACPD facilitation non-competitively. Desensitization of the 1S,3R-ACPD facilitation was not observed. The facilitation was not altered by inhibiting or activating protein kinase C nor was it mimicked by 8-bromo-cAMP, 8-bromo-cGMP or arachidonic acid. However, the facilitation was potentiated by myo-inositol and exhibited heterologous desensitization by 5-HT. The facilitation induced by 1S,3R-ACPD and carbachol was insensitive to removal of external Ca^{2+} . BAPTA-AM, but not nifedipine, eliminated the 1S,3R-ACPD induced facilitation.

It is concluded that 5-HT_{2A} receptors mediate facilitation of the NMDA depolarization through a direct action on cortical pyramidal (projection) neurons. 5-HT_{2A} receptors activate phospholipase C, which in turn generates inositol 1,4,5 triphosphate leading to a rise in intracellular Ca^{2+} . A rise in intracellular Ca^{2+} , whether induced by agonists such as 5-HT, Ca^{2+} ionophores or blockers of Ca^{2+} -ATPase (e.g. thapsigargin) underlies the facilitation. How a rise in Ca^{2+} translates into a larger NMDA response remains to be answered. During aging 5-HT_{2A} receptors are present, but the 5-HT_{2A} facilitation is inhibited due to activation of protein kinase C.

KEYWORDS: Serotonin, 5HT_{2A} receptor, NMDA receptor, neurotransmitter interaction, protein kinase C, neocortex, aging, metabotropic glutamate receptors.

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LIST OF ABBREVIATIONS

1S, 3R-ACPD	1-aminocyclopentane-1S,3R-dicarboxylic acid
2-OH saclofen	3-amino-2-(4-chlorophenyl)-2-hydroxypropylsulfonic acid
5-HT	5-hydroxytryptamine
8-bromo-cAMP	8-bromo-adenosine cyclic 3',5'-hydrogen phosphate monosodium salt
8-bromo-cGMP	8-bromo-guanosine cyclic 3',5'-hydrogen phosphate monosodium salt
8-OH-DPAT	(±)-8-hydroxy-dipropylaminotetralin
aa	amino acid
AA	arachidonic acid
ACh	acetylcholine
ACSF	artificial cerebrospinal fluid
Ag/AgCl	silver/silver chloride
D,L-AP3	2-amino-3-phosphonopropionic acid
D,L-AP4	2-amino-4-phosphonobutyric acid
AMPA	α-2-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionate
ANOVA	Analysis of variance
ATP-ase	adenosine triphosphatase
BAPTA	bis-(o-aminophenoxy)-ethane-N,N,N',N', tetraacetic acid

BAPTA-AM	bis-(0-aminophenoxy)-ethane-N,N,N',N',tetraacetic acid aminomethoxy
CaM	calmodulin
CCH	carbachol
c.f.	confer
DAG	1,2-diacylglycerol
DOI	(±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane
DPAT	8-OH-DPAT
EAA	excitatory amino acid
EC ₅₀	concentration at which 50% of maximal response is observed
EEG	Electroencephalogram
eg.	exempli gratia (for example)
EPSP	excitatory postsynaptic potential
g	gram(s)
GABA	Gamma-aminobutyric acid
GABA _A	GABA-A receptor
GABA _B	GABA-B receptor
Glu	Glutamate
G-protein	guanine nucleotide-binding protein

G α	α subunit of G-protein
hr	hour
IC ₅₀	concentration at which 50% of inhibition is observed
i.e.	id est (that is)
IP ₃	inositol 1,4,5-triphosphate
IP ₃ R	IP ₃ -receptor
IPSP	inhibitory postsynaptic potential
LVFA	low voltage fast activity
M	molar
M-ACSF	modified artificial cerebrospinal fluid
mGluR	metabotropic glutamate receptor
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
mV	millivolt
mRNA	messenger ribonucleic acid
NE	norepinephrine
nM	nanomolar

NMDA	N-methyl-D-aspartate
NMDA-R	NMDA-receptor
OAG	1-oleo-2-acetyl-sn-glycerol
PBS	phosphate buffered saline
PD	phorbol diacetate
PE	phenylephrine
PI	phosphoinositide
PIP ₂	Phosphatidyl inositol 4,5-biphosphate
PKA	protein kinase A
PKC	proten kinase C
PLC	phospholipase C
PLD	phospholipase D
PLA ₂	phospholipase A ₂
PT	pertussis toxin
SEM	standard error of mean
SSC	sodium chloride/sodium citrate
SSRIs	selective serotonin reuptake inhibitors
ST	staurosporine
TFMPP	m-trifluoromethylphenylpiperazine
tRNA	transfer ribonucleic acid

TTX	tetrodotoxin
μg	microgram
μm	micrometre(s)
μM	micromolar
VDCC	voltage-dependent calcium channel
vol/vol	volume/volume

CHAPTER 1

INTRODUCTION

1.1 Statement of the research problem

Serotonin (5-hydroxytryptamine; 5-HT) acting at numerous receptor subtypes has been identified as a neurotransmitter/neuromodulator in a wide variety of physiological and behavioral processes (Aghajanian *et al.*, 1987, 1990; Jacobs, 1991; Jacobs and Fornal, 1991, 1993; Jacobs and Azmitia, 1992; McCormick, 1992). This is not surprising considering the almost ubiquitous distribution of 5-HT containing fibres and terminals throughout the mammalian central nervous system (Jacobs and Fornal, 1993).

Recently, 5-HT has been implicated as a neuromodulator with respect to the excitability of cortical pyramidal neurons (Neuman, 1986; Neuman and Zebrowska, 1992). The term "**neuromodulation**" may be defined as the ability of neurons to alter their electrical properties in response to intracellular biochemical changes resulting from synaptic or hormonal stimulation (Kaczmarek and Levitan, 1987). Generally, modulatory effects involve a second messenger system (Nicoll *et al.*, 1990). Results from both systemic and iontophoretic application of 5-HT antagonists are consistent with 5-HT_{2A} receptors mediating the modulatory activity

of 5-HT on cortical pyramidal neurons (Neuman and Zebrowska, 1992). A possible interaction between glutamate activated N-methyl-D-aspartate (NMDA) receptors and 5-HT receptors was proposed to underlie this process (Neuman and Zebrowska, 1992), but this remains to be confirmed. *In vitro*, 5-HT has been shown to facilitate the NMDA depolarization of neocortical neurons (Nedergaard *et al.*, 1986, 1987; Reynolds *et al.*, 1988). However, with regards to the facilitation, 5-HT receptor characterization and the underlying mechanism were not determined. Therefore, the present investigation was aimed at examining the modulatory role of 5-HT with respect to facilitating the NMDA depolarization. To this end, this thesis has undertaken a quantitative pharmacological study concerning the interaction between 5-HT receptors and NMDA receptors and the underlying mechanism by which 5-HT facilitates the NMDA depolarization *in vitro*. Effects of other neurotransmitters/neuromodulators on the NMDA response have also been examined. In the first part of the thesis, characterization of the 5-HT receptor, regulation of the 5-HT receptor in mediating the interaction, and the underlying mechanism of interaction are examined. In the second part of this thesis, changes in 5-HT_{2A} receptor activity are examined in senescent rats. In the final section of the thesis, the facilitation induced by 5-HT is compared with that induced principally by metabotropic glutamate receptor agonists and also muscarinic cholinergic receptor and α_1 -adrenoceptor agonists.

1.2 5-HT system in the central nervous system

1.2.1 Historical background

5-HT is an endogenous substance, derived from an indole nucleus and is, therefore, classified as an indoleamine. Physiologists of the 19th century were aware of a substance in blood serum that caused constriction of blood vessels and increased vascular tone (Stevens and Lee, 1884; Brodie, 1900). In 1947, Rapport and colleagues identified a serum-borne substance, which caused vasoconstriction and named it "serotonin" (serum tonic). Subsequently, the substance isolated, purified and identified as an active component of their crystalline complex was the chemical 5-hydroxytryptamine (Rapport *et al.*, 1948; Rapport, 1949). Erspamer and Asero (1952) in Italy, had identified a substance found in high concentrations in enterochromaffin cells of the intestinal mucosa in the gut, which they termed "enteramine," that later was found to be identical with serotonin. Serotonin was synthesized chemically (Hamlin and Fisher, 1951) as the indolealkylamine. Following the isolation and synthesis of 5-HT in the early 1950s, there was increasing interest in the physiological function of this indoleamine. Twarog and Page (1953) soon detected 5-HT in the mammalian brain and in subsequent studies 5-HT was proposed to be a neurotransmitter in the central nervous system (Amin *et al.*, 1954; Bogdanski *et al.*, 1956; Brodie and Shore, 1957). The accumulated

evidence suggests that 5-HT fulfils the criteria formulated by Iversen (1975) for a putative neurotransmitter in the central nervous system (Fuxe, 1965; Carlsson *et al.*, 1969). Interest in the physiological role of 5-HT in the central nervous system has preoccupied neurobiologists since that time. Numerous reports show that 5-HT affects various functions of the central nervous system, including sleep (Jouvet, 1967; Koella, 1988), learning and memory (Altman and Normalie, 1988; McEntee and Crook, 1991), motor activity (Gershon and Baldessarini, 1980; Sternbach, 1991; Jacobs and Fornal, 1993), thermoregulation (Myers, 1981), feeding (Blundell, 1977, 1984; Marazziti *et al.*, 1988; Curzon, 1990) sexual behavior (Fernandez-Guasti *et al.*, 1987; Gorzalka *et al.*, 1990), neuroendocrine regulation (Fuller, 1990; Cowen *et al.*, 1990; Van de Kar, 1991) and biological rhythms (Wesemann and Weiner, 1990).

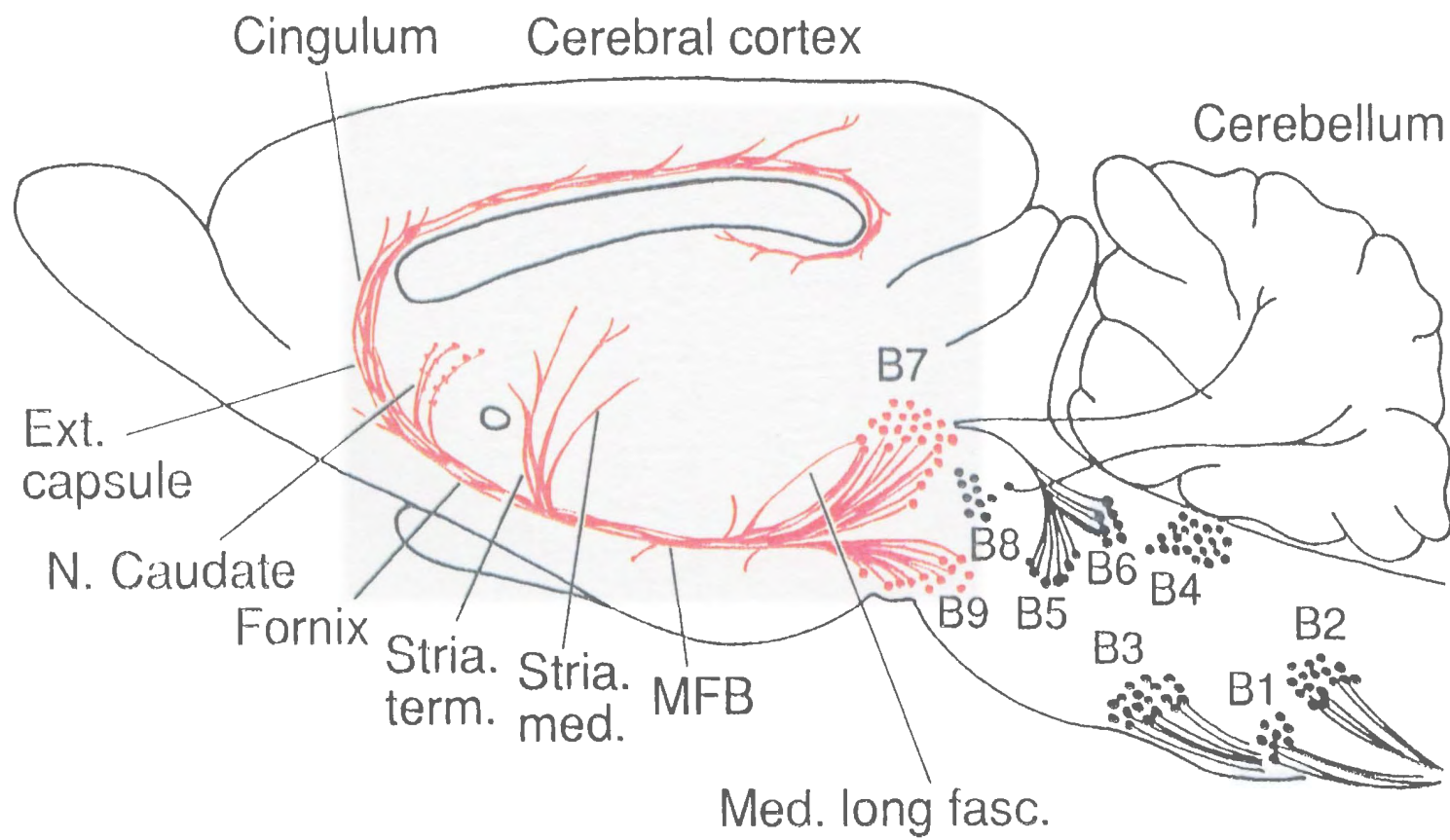
1.2.2 5-HT neurons

The 5-HT system, originally mapped by Dahlstrom and Fuxe (1964), is one of the most extensive monoaminergic systems in the brain as demonstrated by studies in rat, cat and monkey (Dahlstrom and Fuxe, 1964, 1965; Fuxe, 1965; Anden *et al.*, 1966; Fuxe and Jonsson, 1974; Azmitia and Segal, 1978; Lidov *et al.*, 1980; Parent *et al.* 1981; Steinbush, 1981). The 5-HT system consists of a morphologically diverse group of neurons, whose cell bodies are located in the

brain stem raphe nuclei and some regions of the reticular formation, and complex axonal systems which innervate virtually all regions of the central nervous system (Steinbush, 1981; Azmitia and Gannon, 1986; Mulligan and Tork, 1988; Tork, 1990).

However, there are variations in the nomenclature of 5-HT cell groups (Tork, 1990; Jacobs and Azmitia, 1992) out of which the most recent nomenclature of 5-HT cell groups has been adopted in this thesis (Mamounas *et al.*, 1992). Fig. 1 shows that 5-HT neurons are roughly divided into two groups: 1) an ascending group of neurons in mesencephalon and pons which projects rostrally innervating the forebrain, designated raphe dorsalis (B7, and its caudal extension B6), medianus (or nucleus centralis superior; B8 and B5) and B9 cell group; 2) a descending group of neurons in the pons and medulla which projects caudally to the brain stem and spinal cord, designated raphe pallidus (B1), obscurus (B2, B4) and magnus (B3). The raphe dorsalis and medianus project widely to the cerebral cortex (Azmitia and Segal, 1978; Lidov *et al.*, 1980; Steinbush, 1981). The laminar distribution of 5-HT fibres has been described in the cerebral cortex of rat, cat and monkey (see Tork, 1990; Jacobs and Azmitia, 1992; Mamounas *et al.*, 1992, for review). For example, in rat parietal cortex, 5-HT fibres innervate all layers, but form a dense band in the upper part of layer V (Blue *et al.*, 1988). In monkeys, 5-HT innervation was found to have the highest densities in layers I and

Figure 1. Schematic diagram illustrating the distribution of the main 5-HT containing pathways in the rat central nervous system, after Cooper *et al.*, (1991). Abbreviations: Ext. capsule, external capsule; N. Caudate, caudate nucleus; Stria. term., stria terminalis; Stria. med., stria medullaris; MFB, medial forebrain bundle; Med. long. fasc., medial longitudinal fasciculus. For nomenclature of 5-HT cell groups see text.



IV (see Jacobs and Azmitia, 1992, for review). The most highly laminar distribution of all neocortical areas in the monkey is found in the visual cortex (Jacobs and Azmitia, 1992). Recently, morphologically different fine and beaded 5-HT axons have been found arising from the dorsal raphe and median raphe nuclei, respectively (Kosofsky and Molliver, 1987). Pharmacological studies show that only the fine fibres, originating from the dorsal raphe, are destroyed by drugs such as parachloroamphetamine and methylenedioxyamphetamines. Fibres from the median raphe are resistant to this effect suggesting differential vulnerability of the two axon types in cerebral cortex (Mamounas *et al.*, 1992).

1.2.3 Classification and Nomenclature of 5-HT receptors

The pioneering work of early investigators in the 5-HT field found pharmacological evidence for the existence of multiple 5-HT receptors (Rocha e Silva *et al.*, 1953; Gaddum and Hameed, 1954) and laid the foundation for the current understanding of 5-HT pharmacology. Subsequently, Gaddum and Picarelli (1957) demonstrated two distinct 5-HT receptor mediated effects in smooth muscle of the guinea pig ileum. The two presumed receptors causing these effects were labelled M and D receptor based on the ability of morphine and dibenzyline (phenoxybenzamine) to block 5-HT induced contraction of intestinal smooth muscle.

The advent of receptor binding studies confirmed the pharmacological evidence for multiple receptors by revealing the existence of multiple binding sites for 5-HT in rat brain (Peroutka and Snyder, 1979). This methodology allowed a preliminary classification of 5-HT receptors, but identification of subtypes was limited by the poor selectivity of the available agonists and antagonists. Bradley *et al.*, (1986) proposed three major 5-HT receptor classes on the basis of an interaction with receptor agonists. These are 5-HT₁-like, 5-HT₂ and 5-HT₃. 5-HT₁-like was proposed for the heterogeneous group of receptors including the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1C}, 5-HT_{1D} and 5-HT_{1E} which demonstrate a high affinity for 5-HT and 5-carboxyamidotryptamine (5-CT), and are antagonized by methiothepin and methysergide (Pazos *et al.*, 1984; Peroutka, 1986; Heuring and Peroutka, 1987; Leonhardt *et al.*, 1989). 5-HT₂ was proposed for the D (Gaddum and Piccarelli, 1957) receptor and corresponds to the cortical 5-HT₂ binding site (Peroutka and Snyder, 1979). These receptors were characterized by their low affinity for 5-HT and high affinity for the 5-HT antagonists ketanserin, methysergide, mianserin and metergoline (Bradley *et al.*, 1986; Glennon, 1987). The 5-HT₃ receptor corresponds to the M receptor (Gaddum and Piccarelli, 1957). ICS 205 930 and MDL 72222 are high affinity antagonists at 5-HT₃ receptors (Fozard *et al.*, 1979; Fozard, 1984; Richardson *et al.*, 1985). Evidence for four additional 5-HT receptors (5-HT₄ through 5-HT₇) in the CNS has appeared over the last few years

(Shenker *et al.*, 1987; Dumuis *et al.*, 1988; Andrade and Chaput, 1991; Bockaert *et al.*, 1992; Hoyer *et al.*, 1994).

Using molecular biological techniques, more 5-HT receptors have been identified. Therefore, the classification of 5-HT receptors has been modified by the Serotonin Club Receptor Committee and approved by the International Union of Pharmacological Societies (IUPHAR) Commission on Serotonin Nomenclature (Humphrey *et al.*, 1993; Hoyer *et al.*, 1994). The new system classifies 5-HT receptors on the basis of three characteristics: i) operational (drug related), ii) transductional (receptor-coupling) and iii) structural (primary amino acid sequence). Based on these criteria, the currently recognized 5-HT receptors can be subdivided into seven receptor subtypes. Following this classification, 5-HT₂ and 5-HT_{1C} receptors have been renamed as 5-HT_{2A} and 5-HT_{2C}. Table I summarizes the most recent classification of 5-HT receptor subtypes together with their anatomical location and most specific agonists and antagonists (Humphrey *et al.*, 1993, Hoyer *et al.*, 1994; Martin and Humphrey, 1994). In this thesis, the new nomenclature has been adopted.

1.2.4 Localization of 5-HT receptors

Pharmacological, biochemical, functional and *in situ* hybridization studies have revealed the location of multiple of 5-HT receptors in the central nervous

Table I. Classification of 5-HT receptors by modified nomenclature in mammalian brain

Receptor subtype	5-HT _{1A}	5-HT _{1B}	5-HT _{1D}	5-HT _{1E}	5-HT _{1F}
Previous name	-----	-----	-----	-----	5-HT _{1EB} 5-HT ₆
Effector pathways	↓cAMP K ⁺ channel	↓cAMP	↓cAMP	↓cAMP	↓cAMP
Selective agonists	8-OH-DPAT	CP93129	Sumatriptan	-----	-----
Selective antagonists	WAY100135	-----	-----	-----	-----
Structure	421aa human 422aa rat	386aa rat	377/390aa human	365aa human	366aa rat
Receptor subtype	5-HT _{2A}	5-HT _{2B}	5-HT _{2C}	5-HT ₃	5-HT ₄
Previous name	D,5-HT ₂	5-HT _{2F}	5-HT _{1C}	M	-----
Effector pathways	IP ₃ /DAG	IP ₃ /DAG	IP ₃ /DAG	Cation channel	↑cAMP
Selective agonists	α-methyl 5-HT	α-methyl 5-HT	α-methyl 5-HT	2-methyl 5-HT	5MeOT
Selective antagonists	Ritanserin Ketanserin	LY53857 LY53857	Mesulergine Ondansetron	ICS205930	GR113808
Structure	471aa human 471aa rat	479aa rat	458aa human 460aa rat	487aa mouse	-----
Receptor subtype	5-HT _{5A}	5-HT _{5B}	5-HT ₆	5-HT ₇	
Previous name	5-HT _{5α}	5-HT _{5β}	-----	-----	
Effector pathways	Unknown	Unknown	↑cAMP	↑cAMP	
Selective agonists	-----	-----	-----	-----	
Selective antagonists	-----	-----	-----	-----	
Structure	357aa rat	371aa rat	436aa rat	445aa human	

Modified after Humphrey *et al.*, 1993; Martin and Humphrey, 1994; Hoyer *et al.*, 1994.

system (Pazos *et al.*, 1985; Pazos and Palacios, 1985a; Andrade and Chaput, 1990; Aghajanian *et al.*, 1990; Palacios *et al.*, 1992; Peroutka, 1993; Hoyer *et al.*, 1994; Pompeiano *et al.*, 1994; Zifa and Fillion, 1992). Because of the importance and relevance of 5-HT_{2A}, 5-HT_{2C}, and 5-HT_{1A} receptors to this thesis, only these subtypes of 5-HT receptors are reviewed. Localization and distribution of 5-HT_{2A} receptors have been reported for the rat (Pazos *et al.*, 1985) and human brain (Hoyer *et al.*, 1986b; Biegon *et al.*, 1986; Pazos *et al.*, 1987b). Species differences (rat vs human) in receptor localization have been reported by receptor autoradiography. 5-HT_{2A} recognition sites occur at high concentrations in rat neocortex, claustrum, olfactory tubercle, olfactory nucleus and piriform cortex (Pazos *et al.*, 1985; Schotte *et al.*, 1983). In the neocortex, high densities are found in the frontoparietal motor and sensory cortex. Layer V of this area is reported to contain the highest densities of 5-HT_{2A} receptors (Pazos *et al.*, 1985). High densities of recognition sites are also observed in layer III and IV depending upon the cortical area (Pazos *et al.*, 1985). High to intermediate concentrations are found in layer I. Intermediate to low concentrations are observed in all other brain regions (Pazos *et al.*, 1985). The laminar distribution of messenger ribonucleic acid (mRNA) for 5-HT_{2A} receptors in rat brain is consistent with that of the binding sites (Pompeiano *et al.*, 1994). In human brain, 5-HT_{2A} binding sites are present at very high concentration in layer III and V (Hoyer *et al.*, 1986b; Biegon

et al., 1986; Pazos *et al.*, 1987b).

5-HT_{2C} receptors are found at high concentrations in the choroid plexus of rat, mouse and human (Yagaloff and Hartig, 1985; Peroutka, 1986; Mengod *et al.*, 1990; Hoyer *et al.*, 1986b) and low levels in most other regions of the brain including cerebral cortex (Pazos *et al.*, 1985b; 1988) with some notable exceptions. Thus, for example, very high concentrations of 5-HT_{2C} binding sites are found in layers II-III and the deep part of layer V of retrosplenial cortex (Pazos and Palacios, 1985a). Intermediate to low densities of 5-HT_{2C} recognition sites are found in the internal layers of anterior cingulate cortex (Pazos and Palacios, 1985a). The distribution of mRNA for 5-HT_{2C} receptors is in keeping with the binding studies (Molineaux *et al.*, 1989; Hoffman and Mezey, 1989; Pompeiano *et al.*, 1994).

Localization of 5-HT_{1A} receptors has been studied in rat, mouse, guinea pig and human brain (Pazos and Palacios, 1985a; Weaber *et al.*, 1989; Hoyer *et al.*, 1985b; Pazos *et al.*, 1987a). In rat, 5-HT_{1A} receptors are found in high densities in entorhinal cortex, hippocampus, some nuclei of amygdaloid complex, and the dorsal and median raphe, which contain 5-HT cell bodies. Intermediate densities of 5-HT_{1A} binding sites are present in claustrum and olfactory tubercle (Pazos and Palacios, 1985a). Intermediate to low concentrations of 5-HT_{1A} binding sites are present in cerebral cortex and nucleus caudate-putamen. The distribution of

mRNA for 5-HT_{1A} receptors is compatible with that of the binding sites (Pompeiano *et al.*, 1992).

1.2.5 Electrophysiology of 5-HT receptors

Understanding of the electrophysiology of 5-HT has progressed with the development of selective agonists and antagonists for these receptors. Between two and three decades ago, single cell recording from neurons in the cerebral cortex and brain stem indicated that multiple 5-HT receptors were present in the central nervous system (Roberts and Straughan, 1967; Haigler and Aghajanian, 1974). Subsequently, three 5-HT receptor subtypes were proposed to account for the early experimental observations (Aghajanian, 1981; Haigler, 1981). The first subtype facilitated the depolarizing action of glutamate at facial motoneurons (McCall and Aghajanian, 1980) and spinal motoneurons (White and Neuman, 1980). This excitatory action of 5-HT on motoneurons was antagonized by classical 5-HT antagonists such as methysergide, cinanserin and cyproheptadine (Haigler and Aghajanian, 1974; Aghajanian and Wang, 1978; Aghajanian, 1981). The inhibition produced by 5-HT in forebrain regions including neocortical neurons was attributed to the second 5-HT receptor subtype (Reader *et al.*, 1979; Aghajanian, 1981). Classical 5-HT antagonists were not consistently effective in blocking this receptor (Aghajanian, 1981). The third subtype of receptor was the

presynaptic autoreceptor, which was located in the somatodendritic region of 5-HT neurons in the dorsal and median raphe nuclei. Activation of these receptors by 5-HT inhibited 5-HT neurons and as a consequence 5-HT neurotransmission was reduced (Aghajanian, 1981). Classical 5-HT antagonists were ineffective at this receptor. Can the three types of receptors be fit into current understanding of 5-HT receptors and if so which receptors are responsible for each action?

The 5-HT_{2A}, and in some cases the 5-HT_{2C}, receptor subtype most closely approximate the first subtype. Activation of presumed 5-HT_{2A} receptors causes neuronal excitation on motoneurons and pyramidal neurons (VanderMaelen and Aghajanian, 1982; Davies *et al.*, 1987) and agonists at these receptor are often hallucinogenic (Glenon, 1990). At cortical neurons, 5-HT_{2A} receptors enhance cell excitability by: i) a direct membrane depolarization; and ii) modulation of the slow voltage and calcium-activated membrane currents present in these cells (Andrade and Chaput, 1991). The slow depolarization of cortical neurons is associated with a decreased resting K⁺ conductance and reduction of the slow afterhyperpolarization (Davies *et al.*, 1987; McCormick and Williamson, 1989; Araneda and Andrade, 1991; Tanaka and North, 1993). 5-HT_{2A} receptors in the prefrontal cortex are also capable of regulating slow voltage and Ca²⁺ activated currents by membrane depolarization and potentiate the effect of excitatory inputs onto these cells (c.f. Andrade and Chaput, 1991). Classical 5-HT receptor

antagonists block all of the above actions but in most cases the concentrations employed are not appropriate to discriminate between 5-HT_{2A} and 5-HT_{2C} subtypes.

As with pyramidal neurons, stimulation of the presumed 5-HT_{2A} receptors on facial motoneurons evokes a slow depolarization, increases input resistance, and increases excitability, probably through a decrease in resting membrane conductance to potassium (Vandermaelen and Aghajanian, 1980, 1982; Rasmussen and Aghajanian, 1990). The action of 5-HT in the facial nucleus can be blocked by the classical 5-HT antagonists metergoline, methysergide, cyproheptadine and cinanserin (McCall and Aghajanian, 1980). Similar observations with respect to 5-HT_{2A} receptors have been made in nucleus accumbens neurons (North and Uchimura, 1989). In piriform cortex, 5-HT_{2A} receptors are also excitatory, but instead of being located on pyramidal neurons they are found on inhibitory interneurons (Sheldon and Aghajanian, 1990). Thus, activation of 5-HT_{2A} receptors induces inhibitory postsynaptic potentials (IPSPs) on layer II neurons. The IPSPs are in turn blocked by a gamma-aminobutyric acid-A (GABA_A) receptor antagonist bicuculline (Sheldon and Aghajanian, 1990). 5-HT_{2C} receptors are present in high concentration in the piriform cortex where they appear to be located on layer II cells. These 5-HT_{2C} receptors are directly excitatory on layer II neurons in the piriform cortex. When 5-HT_{2C} receptor mRNA is injected into *Xenopus* oocytes, it stimulates Ca²⁺ release which in turn induces a Ca²⁺ dependent chloride current

(Lubbert *et al.*, 1987). 5-HT_{2A} receptor mRNA has the same action in *Xenopus* oocytes suggesting the 5-HT_{2C} receptors on central nervous system neurons are likely to mimic the action of 5-HT_{2A} receptors, although whether this is true in all respects remains to be established. In summary, the electrophysiological evidence is uniformly consistent that 5-HT_{2A} and 5-HT_{2C} receptors are excitatory on neurons, although depending on the type of neurons on which they are found, the net effect of their activation may be excitatory or inhibitory.

In contrast with the 5-HT_{2A} and 5-HT_{2C} receptors, activation of 5-HT_{1A} receptors results in an inhibitory hyperpolarization. The 5-HT_{1A} receptor mediated hyperpolarization has been reported in neurons of the frontal cortex (Araneda and Andrade, 1991), cingulate cortex (Tanaka and North, 1993), hippocampus (Andrade and Nicoll, 1987; Colino and Halliwell, 1987), dorsal raphe (Aghajanian and Lakoski, 1984; Williams *et al.*, 1988), and superior cervical ganglia (Ireland and Jordan, 1987). The 5-HT_{1A} receptor mediated hyperpolarization is consistently associated with an increased potassium conductance (Araneda and Andrade, 1991; Tanaka and North, 1993) dependent on a pertussis toxin sensitive guanylate nucleotide binding protein (Andrade and Nicoll, 1987). 5-HT_{1A} selective agonists including 8-hydroxy-dipropylaminotetralin HBr (8-OH-DPAT) and ipsapirone mimic the inhibitory effect of 5-HT on postsynaptic sites as well as on somatodendritic autoreceptors on dorsal raphe neurons (Andrade and Chaput,

1991). Antagonists at 5-HT_{1A} receptors can block the 5-HT induced hyperpolarization at postsynaptic sites as well as the reduction in excitability on dorsal raphe cell firing (VanderMaelen *et al.*, 1986; Sprouse and Aghajanian, 1987). Thus, subtypes 2 and 3 of Aghajanian (1981) appear to most closely resemble the 5-HT_{1A} receptor subtype. Much less is known regarding the electrophysiological action of the remaining 5-HT receptors found in the central nervous system with the exception of the 5-HT₃ receptor (Hoyer *et al.*, 1994). Unlike other 5-HT receptors, 5-HT₃ receptors have a rapid excitatory action which has been demonstrated in a number of cells including cultured mouse hippocampal and striatal neurons (Yakel and Jackson, 1988). 5-HT₃ receptors are directly coupled to a nonspecific cation channel, i.e. the response is not mediated by a second messenger. It is the direct coupling which allows for the rapid excitatory action. Like 5-HT_{2A} receptors, the 5-HT₄ receptor mediates a membrane depolarization and reduction in afterhyperpolarization, at least in rat hippocampal neurons (Andrade and Chaput, 1991a).

1.2.6 5-HT receptor activated second messengers

It is well established that 5-HT receptors are linked to two major second messenger systems in mammalian central nervous system. The two systems transmitting extracellular signal or receptor activation across the plasma membrane

are the adenylate cyclase/cyclic AMP cascade and phospholipase C/phosphoinositide (PI) hydrolysis cascade (Chuang, 1989). These signals modify cellular systems largely through the common mechanism of phosphorylation (Cooper *et al.*, 1991). Based on second messenger systems, 5-HT receptors can be divided into three categories, those which inhibit (5-HT₁) or stimulate (5-HT₄, 5-HT₆ and 5-HT₇) the adenylate cyclase/cyclic AMP system and those which stimulate phospholipase C/phosphoinositide hydrolysis (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}; Martin and Humphrey, 1994; Hoyer *et al.*, 1994).

Among the 5-HT₁ receptor family, activation of the 5-HT_{1A} receptor subtype has been found to produce inhibition of forskolin stimulated adenylate cyclase in the central nervous system of rat, guinea pig and mouse (see Zifa and Fillion, 1992; Hoyer *et al.*, 1994, for review). However, activation of 5-HT_{1A} receptors has also been shown to couple positively to adenylate cyclase (see Hoyer and Schoeffter, 1991; Zifa and Fillion, 1992, for review). Limited reports suggest 5-HT_{1A} receptors may also influence second messenger processes such as phosphoinositide turnover and liberation of arachidonic acid (Claustre *et al.*, 1988, 1991). The remaining 5-HT receptors (5-HT_{1B} to 5-HT_{1F}) appear to be negatively linked with adenylate cyclase in the central nervous system (Hoyer *et al.*, 1994 and above Refs.).

5-HT_{2A} and 5-HT_{2C} receptors stimulate PI hydrolysis, again demonstrating the similarity between these two receptors (Kendall and Nahorski, 1985; Conn and Sanders-Bush, 1984, 1986, 1987; Hide *et al.*, 1989; Hoyer *et al.*, 1989). 5-HT_{2A} receptors couple to phospholipase C (PLC) through a guanylate nucleotide binding protein (G-protein). When PLC is activated, it metabolizes phosphoinositide 4,5-biphosphate (PIP₂) to produce at least two second messengers, inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG; see Berridge, 1987; Nishizuka, 1988; Chuang, 1989 for review). IP₃ acting at an IP₃ receptor on smooth endoplasmic reticulum releases calcium (Taylor and Richardson, 1991) and DAG activates protein kinase C (Nishizuka *et al.*, 1991). There are other inositol phosphates formed, but whether these are significant for signalling remains to be established. However, the released Ca⁺⁺ activates a number of enzymes which phosphorylate cellular proteins (Kennedy, 1992). How this phosphorylation alters neuronal signal transduction processes is still unclear. Protein kinase C (PKC) is involved in the phosphorylation reactions (Nishizuka *et al.*, 1991; Stabel and Parker, 1993) and regulates receptor function (Roth *et al.*, 1986; Aghajanian, 1990). In cortical slices, activation of 5-HT_{2A} receptors induces a concentration and time related translocation of protein kinase C through a Ca⁺⁺ dependent mechanism (Wang and Friedman, 1990). 5-HT can also stimulate phospholipase A₂ and subsequent release of arachidonic acid in hippocampal and cortical neurons via 5-HT₂ which

presumably are 5-HT_{2A} receptors (Felder *et al.*, 1990). Similarly, 5-HT_{2C} receptor activation in the choroid plexus and the hippocampus leads to the stimulation of PLC and accumulation of inositol phosphates (see Hoyer *et al.*, 1994, for review). Hartig *et al.*, (1990) reported that activation of 5-HT_{2C} receptors also regulates the production of cGMP in the choroid plexus.

5-HT₄, 5-HT₅, 5-HT₆ and 5-HT₇ are present in the central nervous system and except for 5-HT₅, appear to couple positively to adenylate cyclase. The action of 5-HT₅ is not known (see Hoyer *et al.*, 1994, for review). An extensive review of these receptors is beyond the scope of this thesis.

1.2.7 Molecular biology of 5-HT receptors

Molecular biological data have unequivocally confirmed the existence of multiple 5-HT receptors (Boess and Martin, 1994). Indeed, the multiplicity of 5-HT receptor subtypes, both within and between species, has exceeded most of the predictions that might have been made on the basis of pharmacological data (Peroutka, 1994). So far, fourteen subtypes of 5-HT receptors including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT₃, 5-HT₄, 5-HT_{5A}, 5-HT_{5B}, 5-HT₆ and 5-HT₇ have been cloned (see Boess and Martin, 1994, for review). However, only an overview of the most relevant subtypes, 5-HT_{2A}, 5-HT_{2C} and 5-HT_{1A} will be presented.

All three cloned receptors are single subunit proteins and members of the G-protein receptor family. This family of receptors is characterized by the presence of seven transmembrane domains and by the ability to activate G protein dependent processes such as activation or inhibition of adenylate cyclase (i.e. 5-HT_{1A}), or stimulation of phosphoinositide hydrolysis (see Julius *et al.*, 1990; Boess and Martin, 1994; Martin and Humphrey, 1994, for review). Gene structure and primary amino acid sequence comparisons indicate that the 5-HT_{1A} receptor has evolved from the family of adrenergic receptors, whereas the 5-HT_{2A} and 5-HT_{2C} receptors define a separate gene family (Huang and Julius, 1991). The 5-HT_{2A} receptor shows homology to the 5-HT_{2C} receptor (53% overall sequence identity, 80% identity in transmembrane domain), whereas the 5-HT_{2A} receptor is only 41% identical to the 5-HT_{1A} receptor (Julius *et al.*, 1990; Saltzman *et al.*, 1991; Boess and Martin, 1994). The human 5-HT_{2A} receptor is also homologous with the rat receptor (87%), with the highest amino acid homology (98%) found within the transmembrane regions (Julius *et al.*, 1990). The amino acid sequence for both the 5-HT_{2A} and 5-HT_{2C} receptors reveals the presence of numerous serine/threonine residues which may be possible targets for phosphorylation (Boess and Martin, 1994) and potential sites at which receptor activity can be controlled.

1.2.8 Regulation of 5-HT_{2A} receptors

Since the present thesis deals mainly with the 5-HT_{2A} receptor, this section focuses only on this subtype. 5-HT_{2A} receptors are regulated by a large number of exogenous and endogenous factors (Roth *et al.*, 1990; Leysen and Pauwels, 1990). Like classical G-protein coupled receptors (i.e. β -adrenoceptors), 5-HT_{2A} receptors are regulated by agonist induced down-regulation. For example, exposure to 5-HT_{2A} receptor agonists or partial agonists reduces binding to the 5-HT_{2A} receptor (Leysen *et al.*, 1989; McKenna *et al.*, 1989; Pranzatelli, 1991). However, unlike other receptors, 5-HT_{2A} receptors undergo down-regulation following chronic exposure to antagonists (Leysen and Pauwels, 1990). Such down-regulation is evidenced not only by a decrease in 5-HT_{2A} receptor density, but also by a subsensitive phosphoinositide response (Zifa and Fillion, 1992). Chronic administration of many antidepressants causes down-regulation of the 5-HT_{2A} binding sites (Peroutka and Snyder, 1980; Kendall and Nahorski, 1985; Mizuta and Segawa, 1989) which is accompanied by decreased IP₃ production (see Zifa and Fillion, 1992 for review). On the other hand, administration of electroconvulsive shock, an effective treatment for depression, increases the number of 5-HT_{2A} receptors (Kellar *et al.*, 1981). 5-HT_{2A} receptors may also be down-regulated by treatment with many antipsychotic agents which bind to 5-HT_{2A} receptors (Roth *et al.*, 1990; Roth and Ciaranello, 1991).

Aging also appears to alter regulation of 5-HT_{2A} receptors. Thus, 5-HT_{2A} labelled binding sites decrease in number during aging in both rat (Battaglia *et al.*, 1987; Gozlan *et al.*, 1990) and human brain (Wong *et al.*, 1984; Marcusson *et al.*, 1984; Reynolds *et al.*, 1984; Gross-Isseroff *et al.*, 1990). Moreover, coupling between 5-HT_{2A} receptors and G-proteins is also altered with aging (Robson *et al.*, 1993). These intriguing findings raise the question as to whether age related cognitive deficits observed in both rats and humans might result in part from altered 5-HT_{2A} receptor activity.

1.2.9 Role of 5-HT in neuropsychiatric disorders

In recent years, evidence has emerged to suggest that altered 5-HT receptor mediated function is associated with the pathophysiology of diverse psychiatric conditions (Siever *et al.*, 1991; Sandyk, 1992). Evidence implicating abnormalities of 5-HT function have been reported for disorders including: 1) Alzheimer's disease (Cross *et al.*, 1986; Reynolds *et al.*, 1984; Cross, 1990, Cheng *et al.*, 1991); 2) Parkinsonism (Cheng *et al.*, 1991); 3) suicidal behaviour (Mann *et al.*, 1990; Coccaro and Astill, 1990); 4) schizophrenia (Kahn and Davidson, 1993); 5) depression (Cowen, 1990, 1993; Plaznik *et al.*, 1989; Meltzer, 1990); 6) anxiety (Iverson, 1984; Gardner, 1986; Kahn *et al.*, 1988; Nutt and George, 1990; Hamon, 1994; Bison and Bison, 1994); and 7) obsessive compulsive disorders (Zak *et al.*,

1988; Insel *et al.*, 1990; Murphy *et al.*, 1992). Furthermore, drugs affecting 5-HT transmission or 5-HT receptors are widely used for a number of clinical conditions. For example, selective serotonin reuptake inhibitors (SSRIs) are effective in the treatment of major depression and obsessive-compulsive disorder (see Fuller, 1991, Blier and Montigny, 1994, for review). 5-HT_{2A} receptor antagonists such as mianserin and ritanserin appear to be effective antidepressants (Sleight *et al.*, 1991; Bersani *et al.*, 1991). 5-HT_{2A} receptor antagonists, either alone or in combination with a neuroleptic, reduce negative symptoms in patients with schizophrenia (Gelders, 1989; Castelao *et al.*, 1989; Leysen *et al.*, 1993). Thus, understanding the pharmacology of 5-HT receptors has potentially important therapeutic implications.

1.2.10 5-HT and alteration of neocortical excitability

***In vivo* studies**

In keeping with the widespread distribution of 5-HT containing fibres in the mammalian cortex (see section 1.2.2), 5-HT has been observed to profoundly alter cortical excitability. Epitomizing this is the growing body of evidence that 5-HT mediates atropine resistant cortical desynchronization (low voltage fast activity) (Vanderwolf and Baker, 1986; Vanderwolf, 1988; Vanderwolf *et al.*, 1989).

In urethane anaesthetized rats, noxious stimulation reliably elicits cortical desynchronization, providing 5-HT transmissive processes remain functional. Depletion of 5-HT, blockade of dorsal raphe unit activity, or administration of 5-HT_{2A} receptor antagonists prevents induction of cortical desynchronization by noxious stimulation (Neuman, 1986; Neuman and Thompson, 1989; Thompson *et al.*, 1991). This action of 5-HT is mediated cortically, since iontophoretic release of 5-HT_{2A} receptor antagonists onto layer V pyramidal neurons eliminates cortical desynchronization in response to noxious stimulation. Moreover, the underlying cellular event responsible for desynchronization, i.e. transformation of unit activity from burst-pause to continuous (Creutzfeldt and Houchin, 1974), is also abolished by such treatment (Neuman and Zebrowska, 1989, 1992). These findings are in accord with a high density of 5-HT_{2A} binding sites in layer V of the cortex (Pazos *et al.*, 1985; Appel *et al.*, 1990; Pompeiano *et al.*, 1994) and the observations that dorsal raphe 5-HT fibres are in register with 5-HT_{2A} receptors in layer V (Blue *et al.*, 1988). The role of 5-HT_{2A} receptors in cortical desynchronization appears to be state dependent. In urethane anaesthetized rats, the desynchronization is readily blocked by 5-HT_{2A} receptor antagonists (Neuman and Zebrowska, 1992). The slow waves observed in animals anaesthetized with urethane resemble the slow wave activity observed during sleep. Moreover, 5-HT_{2A} receptor antagonists readily prolong slow wave sleep in both rats and humans which is in keeping with 5-HT_{2A}

receptor antagonists preventing cortical desynchronization (Neuman and Zebrowska, 1992). However, in awake freely moving animals, 5-HT_{2A} receptor antagonists do not prevent cortical desynchronizations (Vanderwolf, 1988). Indeed, the 5-HT receptor(s) mediating this response in awake animals remains to be determined.

NMDA receptors are involved in generating spontaneous bursting at cortical neurons (Flatman *et al.*, 1983). These spontaneous bursts of spikes produced by layer V pyramidal neurons are blocked by D-2-amino-5-phosphonopentanoic acid (AP5), an NMDA receptor antagonist, and promoted by iontophoresis of NMDA (Armstrong-James and Fox, 1988; Fox and Armstrong-James, 1986). Both anatomical and functional evidence suggest that NMDA receptor mediated input to layer V pyramidal neurons arises from the intralaminar nucleus of the thalamus (Fox and Armstrong-James, 1986). This input exhibits a pacemaker role to elicit bursting activity in cortical neurons (Fox and Armstrong-James, 1986). The effect of noxious stimulation on cortical neurons appears to result from enhancement of NMDA responses which is manifest as an increase in the number of spikes per burst. However, the mechanism(s) responsible for 5-HT induced alteration of cortical unit activity remains to be established.

***In vitro* studies**

5-HT typically depolarizes cortical layer V neurons with hyperpolarization observed less frequently in rat, guinea pig and human neocortex (Davies *et al.*, 1987; Araneda and Andrade, 1991; Tanaka and North, 1993; McCormick and Williamson, 1989). The depolarization involves 5-HT_{2A} receptors and results from a decrease in K⁺ conductance whereas the hyperpolarization involves 5-HT_{1A} receptors and results from an increased K⁺ conductance. The depolarization found with 5-HT undergoes homologous desensitization (Araneda and Andrade, 1991). 5-HT also blocks the slow afterhyperpolarization (AHP) at high concentrations. Both the depolarization and the slow AHP are mimicked by phorbol esters which activate PKC (Araneda and Andrade, 1991). This is consistent with the evidence that activation of 5-HT_{2A} receptors stimulates PI hydrolysis and the resulting second messenger cascade (Berridge, 1987; Chuang, 1989). 5-HT_{2A} receptor antagonists block the depolarizing response revealing an underlying hyperpolarization (Araneda and Andrade, 1991). An unmasking of inhibition by 5-HT_{2A} antagonists is consonant with *in vivo* findings (Lakoski and Aghajanian, 1985; Neuman and Zebrowska, 1992).

1.3 Excitatory amino acid receptors - an overview

Excitatory amino acid (EAA) receptors are the major class of excitatory neurotransmitter receptors in the vertebrate central nervous system (Collingridge and Lester, 1989; Monaghan *et al.*, 1989; Barnes and Henley, 1992; Hollmann and Heinemann, 1994). They are membrane spanning proteins that mediate the stimulatory actions of glutamate and possibly other related endogenous amino acids (Barnes and Henley, 1992). EAA receptors play an important role in many neuronal functions in the central nervous system (see Monaghan *et al.*, 1989; Collingridge and Lester, 1989; Schoepp *et al.*, 1990; Schoepp and Conn, 1993; Nakanishi, 1992; McBain and Mayer, 1994 for review). The diverse functions of EAA neurotransmission are mediated by a variety of EAA receptors that are classified into two distinct groups: 1) ionotropic types; and 2) metabotropic types (Collingridge and Lester, 1989; Monaghan *et al.*, 1989; Schoepp *et al.*, 1990). The ionotropic receptor can be further subdivided into NMDA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and kainate receptors. The AMPA receptor is also activated by quisqualate and was formerly called the quisqualate receptor (Collingridge and Lester, 1989; Watkins *et al.*, 1990). Both AMPA and kainate receptors are ligand-gated, cation specific ion channels (Masu *et al.*, 1993) composed of several subunits similar to the well characterized nicotinic and GABA_A receptors (see Gasic and Hollmann, 1992; Hollmann and Heinemann, 1994

for review).

The metabotropic glutamate receptors (mGluRs) are G-protein linked receptors which activate intracellular second messengers (Schoepp *et al.*, 1990; Schoepp and Conn, 1993; Hollmann and Heinemann, 1994). The mGluRs form a family of at least seven subtypes termed mGluR1-mGluR7 which differs in sequence and signal transduction mechanisms (Masu *et al.*, 1991, 1993; Tanabe *et al.*, 1992, 1993; Abe *et al.*, 1992; Okamoto *et al.*, 1994; Nakanishi *et al.*, 1994). For example, mGluR1 and mGluR5 subtypes couple positively to PLC-PI pathway, whereas the other five subtypes are linked to the inhibition of the cAMP cascade. These receptor subtypes show differences in regional distributions in the brain, e.g. the mGluR1 is abundantly present in cerebellum, whereas mGluR5 is found in cerebral cortex and hippocampus (see Masu *et al.*, 1993; Hollmann and Heinemann, 1994 for review).

An additional class of EAA receptors that is sensitive to the glutamate analogue L-2-amino-4-phosphonobutyric acid (L-AP4) has been reported (Collingridge and Lester, 1989; Watkins *et al.*, 1990). This L-AP4 sensitive receptor may be a presynaptic autoreceptor (Williams *et al.*, 1991).

1.3.1 NMDA receptors

The NMDA receptor complex contains a number of distinct recognition

sites for endogenous and exogenous ligands. These include binding sites for glutamate and the co-agonist glycine, a regulatory or allosteric modulatory site which binds polyamines, a voltage dependent Mg^{2+} binding site, an inhibitory divalent cation site that binds Zn^{2+} and a site in the ion channel which binds open channel blockers such as phencyclidine, ketamine and MK 801 (see Barnes and Henley, 1992; McBain and Mayer, 1994, for review). The NMDA receptor gated ion channel has characteristics that clearly distinguish it from other EAA receptor associated channels (Monghan *et al.*, 1989). The ion channel is permeable to Na^+ , K^+ and Ca^{2+} and is blocked by Mg^{2+} in a voltage dependent manner (Nowak *et al.*, 1984; Ascher and Nowak, 1988). The influx of Ca^{2+} that accompanies receptor activation is believed to be involved in many cellular responses mediated by NMDA receptors, including the generation of long term potentiation(LTP) (Nicoll *et al.*, 1988; Collingridge and Lester, 1989). The Mg^{2+} blockade is relieved by membrane depolarization conferring on the current-voltage relationship a negative slope region. The voltage dependent Mg^{2+} blockade of the NMDA receptor channel is thought to be a key switch in controlling the induction of LTP during tetanic stimulation (Madison *et al.*, 1991).

Molecular cloning of the NMDA receptor has identified a subunit termed NMDAR1 and four additional subunits NMDAR2A-2D (Moriyoshi *et al.*, 1991; Nakanishi, 1992; Monyer *et al.*, 1992; Kutsuwada *et al.*, 1992; Sugihara *et al.*,

1992; Ishii *et al.*, 1993). NMDAR1 is the key subunit that possesses all properties of the NMDA receptor gated channel complex, including agonist and antagonist selectivity, glycine modulation, voltage dependent Mg^{++} blockade, Ca^{++} permeability and inhibition by Zn^{++} (Moriyoshi *et al.*, 1991; Sugihara *et al.*, 1992; Karp *et al.*, 1993). NMDAR2A-2D subunits have no channel activity in the homomeric configuration but potentiate the NMDA receptor activity when combined with NMDAR1 (Ishii *et al.*, 1993). The NMDAR1 mRNA is ubiquitously expressed in almost all neuronal cells throughout the brain whereas the mRNA for the NMDAR2 subunits show overlapping but different expression patterns in various brain regions (Moriyoshi *et al.*, 1991; Ishii *et al.*, 1993). Functionally distinct NMDA receptor subtypes are heteromeric assemblies of NMDAR1 with NMDAR2 subunits (Masu *et al.*, 1993). NMDA receptor subunits contain consensus phosphorylation sites for protein kinases at the cytoplasmic domain (Masu *et al.*, 1993).

Based on binding studies, at least three distinct subclasses of NMDA receptor have been proposed (Ebert *et al.*, 1991; Monghan and Anderson, 1991; Monghan and Beaton, 1991). These are as follows: 1) the striatal or agonist preferring NMDA receptor, 2) the thalamic or antagonist preferring NMDA receptor, and 3) the cerebellar class of NMDA receptor (Barnes and Henley, 1992). However, the recent isolation and sequencing of different molecular species (see

above) of NMDA receptor suggest that at least two pharmacologically distinct types exist in the central nervous system (Stone, 1993); the NMDA-1 receptors present in the spinal cord and cerebellum and NMDA-2 receptor present in the neocortex, hippocampus, and striatum (Stone, 1993).

1.3.2 Modulation of NMDA receptors

The NMDA receptor complex is subject to modulation by : i) ligands binding to the NMDA receptor complex, and ii) transmitters, including glutamate which binds with other receptors.

Allosteric modification of the NMDA receptor complex occurs as a response to ligand binding, and as such represents a major target for drug action, e.g. glycine (Thomson, 1989) and the polyamines (Williams *et al.*, 1991). Glycine has been shown to enhance the effects of NMDA in both electrophysiological (Johnson and Ascher, 1987; Thomson, 1989) and biochemical studies (see Reynolds, 1990 for review). It has been demonstrated that nanomolar concentrations of glycine enhance the frequency of NMDA receptor channel opening in a strychnine insensitive manner (Johnson and Ascher, 1987) suggesting a distinct glycine binding site. Glycine has been reported to prevent desensitization of the NMDA receptor during prolonged exposure to agonists (Mayer *et al.*, 1989; Vyklícky *et al.*, 1990), and by an acceleration of recovery of the receptor from its

desensitized state. It is now well established that glycine acts as a co-agonist at the NMDA receptor complex (see Thomson, 1990; McBain and Mayer, 1994, for review).

The polyamine recognition site has been implicated as a novel activator or positive allosteric modulator of the NMDA receptor complex (Reynolds, 1990; Williams *et al.*, 1991). The endogenous polyamines spermine and spermidine increase the binding of open channel blockers and enhance NMDA elicited currents in cultured neurons (Williams *et al.*, 1991a).

There is now unequivocal evidence that transmitters acting at non-NMDA receptors profoundly influence the responsiveness of glutamate mediated synaptic transmission at NMDA receptors (Reynolds, 1990). This form of modulation is mediated by transmitter activated second messengers. Examples of neurotransmitter modulation of NMDA responses include cholinergic (Markram and Segal, 1990, 1992), noradrenergic (Segal *et al.*, 1991; Mouradian *et al.*, 1991), serotonergic (Nedergaard *et al.*, 1986, 1987; Reynolds *et al.*, 1988), metabotropic glutamatergic (Aniksztejn *et al.*, 1991, 1992; Harvey and Collingridge, 1993), GABA-ergic (Walden *et al.*, 1989), and peptidergic modulation (Monnet *et al.*, 1990). However, it is still unclear as to how the activated second messengers account for the modulation in each case. There may be one or more common pathways activated by second messenger systems which induce facilitation of the

NMDA response (see below). McCall and Aghajanian (1979) first obtained evidence for the modulatory interaction of 5-HT and norepinephrine (NE) with glutamate (likely acting at NMDA receptors) at facial motoneurons. They found that 5-HT and norepinephrine alone had no detectable effect on firing, whereas they facilitated glutamate induced excitation. Similarly, 5-HT and NE were found to have no detectable effects on the spontaneous firing of spinal motoneurons, yet they greatly enhanced the glutamate induced excitation of motoneurons suggesting a modulatory interaction among these neurotransmitters (White and Neuman, 1980; Neuman and White, 1982).

In neocortical neurons, 5-HT alone has been reported to have little or no effect on membrane potential or input resistance of layer V pyramidal neurons and yet the NMDA induced depolarization and underlying inward current were enhanced by 5-HT (Nedergaard *et al.*, 1986, 1987; Reynolds *et al.*, 1988; Mally *et al.*, 1991). 5-HT mediated facilitation is selective for NMDA receptors in rats (Reynolds *et al.*, 1988), but not in cats, where the quisqualate induced depolarization is also enhanced (Nedergaard *et al.*, 1987). 5-HT mediated facilitation is blocked by the 5-HT antagonist cinanserin in cat (Nedergaard *et al.*, 1987). The enhancement is not only observed with exogenous NMDA but the NMDA component of the EPSP is also facilitated by 5-HT (Reynolds *et al.*, 1988; Read *et al.*, 1990). With regard to mechanism, 5-HT mediated facilitation of facial

motoneurons was initially attributed to the blockade of a resting K^+ conductance (McCall and Aghajanian, 1979). However, this mechanism is unlikely to operate in neocortical neurons, since 5-HT mediated facilitation was observed in the presence of potassium channel blockers tetraethylammonium, 4-aminopyridine and cesium and under voltage clamp conditions (c.f. Reynolds *et al.*, 1988). Furthermore, the 5-HT mediated facilitation is suggested to be a direct effect, since 5-HT enhances the NMDA induced inward current in the presence of tetrodotoxin (Reynolds *et al.*, 1988). Consistent with these findings, 5-HT facilitation of cat pyramidal neurons takes place in the presence of tetrodotoxin, tetraethylammonium and Mg^{++} depletion (Nedergaard *et al.*, 1987).

In the hippocampus, acetylcholine (ACh) and carbachol acting on muscarinic cholinergic receptors and norepinephrine acting on α_1 -adrenoceptors also facilitate the NMDA response (Markram and Segal, 1990, 1992; Segal *et al.*, 1991). Moreover, 1S,3R-ACPD, an agonist at mGluR (see section 1.3) potentiates the NMDA induced depolarization in spinal motoneurons (Birise *et al.*, 1993), and hippocampal neurons (Aniksztejn *et al.*, 1991, 1992; Harvey and Collingridge, 1993).

The PI pathway has been implicated as being responsible for the potentiation of NMDA responses in hippocampal neurons (Segal *et al.*, 1991; Markram and Segal, 1992; Harvey and Collingridge, 1993). In particular, the IP_3 induced rise

of intracellular Ca^{2+} is thought to be of importance (Markram and Segal, 1992; Harvey and Collingridge, 1993). Extracellular Ca^{2+} may also contribute to the potentiation of NMDA responses by cholinergic and mGluR agonists in hippocampal neurons (Markram and Segal, 1991, 1992; Harvey *et al.*, 1991; Harvey and Collingridge, 1993).

1.4 Rationale and objectives

The foregoing synopsis draws attention to the many pieces of evidence indicating a physiologically significant interaction between 5-HT and other neuromodulators on the one hand and NMDA receptors on the other. However, many questions remain to be answered. The present investigation was aimed at resolving the following questions regarding the interaction between NMDA and 5-HT and other G-protein coupled receptors in rat neocortex: **i)** which is the subtype of 5-HT receptor that mediates facilitation of the NMDA depolarization? **ii)** is this a direct effect of 5-HT and if so what is the mechanism underlying the enhancement? **iii)** are there significant changes in the 5-HT response with aging? **iv)** do other G-protein coupled receptors whose activation enhances the NMDA depolarization share a common mechanism of action? In light of the evidence reviewed above, the following working hypotheses are proposed:

A. 5-hydroxytryptamine selectively facilitates N-methyl-D-aspartate receptor induced depolarization of neocortical pyramidal (projection) neurons by activating 5-HT_{2A} receptors. Stimulation of 5-HT_{2A} receptors activates phosphatidylinositol hydrolysis and as a consequence of this, the N-methyl-D-aspartate depolarization is enhanced.

B. Facilitation of the N-methyl-D-aspartate response by 5-hydroxytryptamine in neocortical neurons is likely to decline with aging due to a loss of 5-HT_{2A} receptors.

C. Like 5-HT_{2A} receptors, metabotropic glutamate receptors, muscarinic cholinceptors and α_1 -adrenoceptors enhance the N-methyl-D-aspartate depolarization as a result of phosphatidylinositol hydrolysis.

Specific objectives

The specific objectives of this investigation are as follows:

1. To develop a quantitative pharmacological assay to investigate the interaction between 5-HT and NMDA receptors in a rat neocortical slice preparation.

2. To characterize the 5-HT receptor subtype(s) mediating the NMDA facilitation.
3. To study in detail the cellular mechanism by which 5-HT achieves its modulatory action.
4. To investigate the interaction between 5-HT and NMDA in senescent rats and compare these results to those found in young adult rats.
5. To explore the differences between 5-HT and other G-protein coupled neurotransmitter receptors with respect to the mechanism by which these agents facilitate the NMDA depolarization.

CHAPTER 2

5-HT FACILITATES NMDA INDUCED DEPOLARIZATION: RECEPTOR CHARACTERIZATION

2.1 Introduction

In order to characterize the interaction between 5-HT and the depolarization of cortical neurons induced by NMDA, a stable pharmacological assay system was required. One approach would have been to use microelectrode recording from pyramidal neurons (c.f. Reynolds *et al.*, 1988). However, a shortcoming of this method is the time consuming nature of constructing dose-response curves for pharmacological analysis. Moreover, stable long term recordings would be necessary in order to reach equilibrium with low concentrations of antagonists. Finally, due to receptor desensitization (Araneda and Andrade, 1991), a large number of intracellular recordings would be required.

An alternate approach to microelectrode recording was the use of "grease-gap" recording from cortical wedges (Harrison and Simmonds, 1985; Burton *et al.*, 1987, Brugger *et al.*, 1990). This method proved quite convenient to quantitate receptor mediated effects. The method relies on the fact that current induced in the dendrites/cell body by an agent such as an EAA agonist can be measured as a voltage drop between the dendrite/cell body compartment and the axon

compartment if the resistance between the two compartments is increased by using sucrose (sucrose-gap) or silicon grease (grease-gap; Harrison and Simmonds, 1985). The responses recorded using the grease-gap technique depends on the neurons which project through the barrier and the density of the receptors on those neurons (Harrison and Simmonds, 1985). However, one of the disadvantages of this method is that small changes induced by an agonist (e.g. 5-HT_{1A}) in those neurons may not yield a recorded potential difference (Mally *et al.*, 1991). This method allows electrophysiological measurement from a large population of cortical neurons, which is convenient for pharmacological analysis. Using this method, the 5-HT receptor mediating facilitation of the NMDA depolarization was characterized.

2.2 Methods

Procedures followed guidelines of the Canadian Council on Animal Care and the Institutional Animal Care Committee at Memorial University.

2.2.1 Animals

Male Sprague-Dawley rats 100-300 g were purchased from Charles River, Montreal, Quebec. The animals were communally housed in cages (5 per cage) at the animal care unit of the Health Sciences Centre, Memorial University of Newfoundland and kept on a 12 hour light/12 hour dark cycle with controlled humidity and temperature. Food (Purina Rat Chow) and water were provided *ad libitum*.

2.2.2 Wedge preparation and recording

Neocortical wedges from sensorimotor cortex were prepared, with slight modification, as described by Harrison and Simmonds (1985). Rats were anaesthetized with ether and killed with a heavy blow on the neck. The brain was rapidly removed into ice cold modified artificial cerebrospinal fluid (M-ACSF; for composition of M-ACSF, see section 2.2.3). The tissue was then transferred dorsal side uppermost to a filter paper lying on an ice cold surface. The brain was blocked, blotted dry and fixed at its caudal end to a tissue holder using

cyanoacrylate glue (Instant Krazy Glue). Coronal slices 500 μm thick were cut at 0-4°C using a Vibroslice (Campden Instrument Ltd., U.K.). The slices were transferred to an incubation chamber containing M-ACSF at room temperature bubbled with 95% O₂ and 5% CO₂. Six to 7 slices were retained starting slightly anterior to bregma (Paxinos and Watson, 1986) and proceeding caudally. Following 30 min incubation at room temperature (20 to 24 °C) in M-ACSF, the medium was replaced with normal ACSF (see section 2.2.3).

After an additional 30 to 45 min incubation in normal ACSF, a slice was transferred to a petri dish containing ACSF, and divided on the midline. As shown in Fig. 2, a wedge of sensorimotor cortex (1.5 mm wide at the pial surface and 1 mm wide at the corpus callosum) was prepared and mounted in a two compartment recording bath (Harrison and Simmonds, 1985). The larger part of the wedge containing the cell bodies was placed in one compartment while the corpus callosum, containing the projecting axons, was placed in the other (Fig. 3). The two compartments were separated by a grease seal (high vacuum silicone grease, BDH) and each was perfused separately at 2 ml/min via a peristaltic pump (Gilson, Miniplus 2, U. K. or Masterflex Pump Cole-Parmer, USA). The pump and solution reservoirs were electrically isolated from the bath using a dropper assembly to reduce noise. Agonist induced depolarization of the cell bodies was recorded with respect to the corpus callosum using Ag/AgCl electrodes (HLA-003,

Figure 1. Photomicrograph of a coronal section of the brain at the frontal level. The lines indicate the approximate position of the cuts made to produce wedges containing neocortex and corpus callosum. Wedges designated "A" and "B" were used for the majority of experiments. Scale bar is 10 mm.

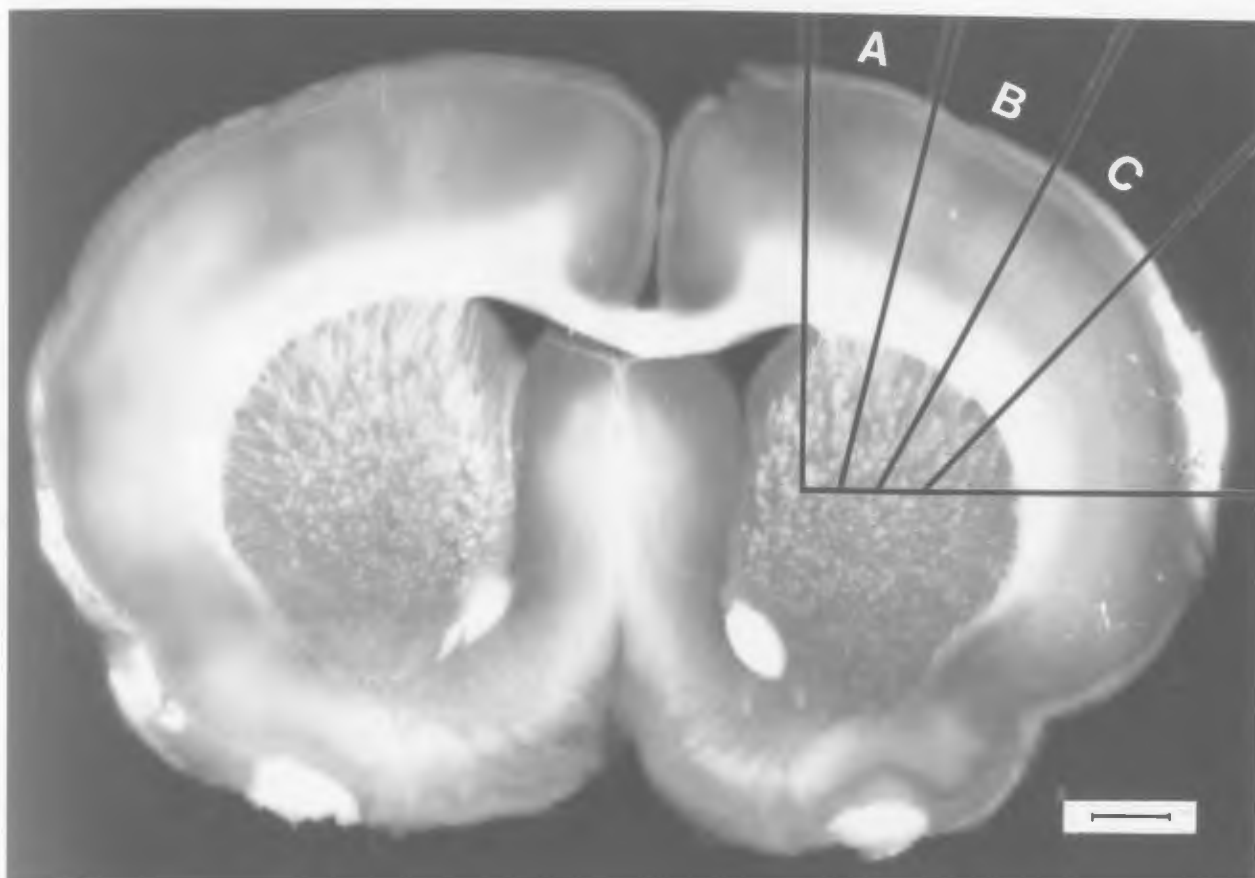
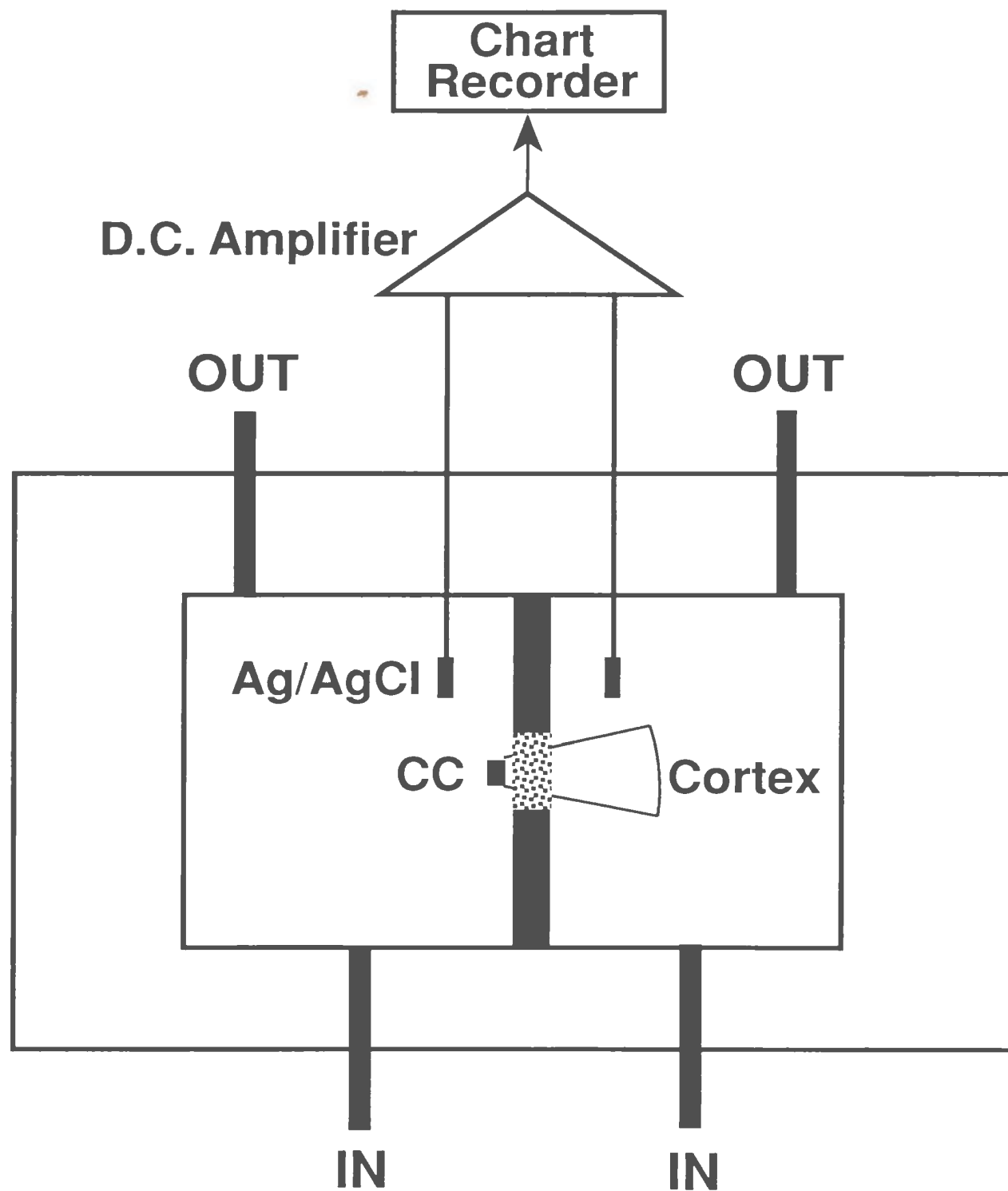


Figure 3. Schematic diagram of a neocortical wedge positioned in the two compartment grease gap recording chambers. CC: corpus callosum. For details see text.



Axon Instruments Inc., USA), embedded in 3% agar containing 1 M NaCl. The electrodes were connected to a high impedance amplifier (Intronix Technologies Corporation, Canada), filtered (0.2 Hz, 3 db down) and the recorded potential displayed on a Grass or Gould chart recorder. The filter was such that only slow depolarizations would be recorded. Depolarization of cortical neurons was recorded as a negative voltage deflection relative to corpus callosum. For clarity, potential deflections are shown as negative upward.

2.2.3 Composition of ACSF

Normal ACSF had the following composition (mM): NaCl 126, KCl 3.5, CaCl_2 2, MgCl_2 1.3, NaH_2PO_4 1.2, NaHCO_3 25, glucose 11. In modified ACSF (M-ACSF), NaCl was replaced by iso-osmotic sucrose (252 mM). This improves slice viability by protecting neurons from Cl^- toxicity (Agahjanian and Rasmussen, 1989). ACSF was gassed with 95% O_2 /5% CO_2 and had a pH of 7.4. Except where otherwise stated, normal ACSF was used throughout.

2.2.4 Drug application

Except as noted, drugs were dissolved in ACSF and applied via a 3 way tap system to the cell body containing compartment. Agonists were applied for 2 min at 20 min intervals unless otherwise noted. To reduce oxidation, 5-HT was diluted

in ACSF just prior to application. Antagonists were administered 30 to 40 min before addition of non-EAA agonists. Drug concentrations were calculated as the salt. Stock solutions were kept frozen until use.

2.2.5 Analysis of data

Depolarization amplitude was used to quantitate drug responses. The amplitude of agonist evoked potentials varied from one wedge to another, likely reflecting, in part, the effectiveness of the grease seal in isolating the compartments. Accordingly, each treatment in a wedge was normalized to percent of control, i.e. $[(\text{Treatment}/\text{Control}) \times 100]$. Graphical analysis (Sokal and Rohlf, 1969) revealed that the frequency distribution of agonist induced responses was not normally distributed unless the data were first transformed to a logarithmic scale (c.f Fleming *et al.*, 1972). The normalized data were therefore transformed for analysis of geometric means. Repeated measures (control and treatment data from the same wedge) were analyzed by paired t tests. Multiple planned comparisons were analyzed by one-way analysis of variance (Instat, GraphPad Software) followed by the Bonferroni test if the F value was significant. The Bonferroni test ensures p values reflect experimentwise error rates. Data are presented as the antilog of the geometric mean. The standard error of the mean of logarithmic data is not symmetrical when retransformed so the larger value is presented. The "n"

value reported in figures and tables refers to number of wedges. Differences between means with "p" values less than 0.05 were considered significant. EC_{50} and IC_{50} values were determined from the logistic dose response curves fitted using the Table Curve (Jandel Scientific, USA).

2.2.6 Drugs

The following drugs were used: carbamylcholine chloride, 5-hydroxytryptamine bimalate salt (5-HT), kainic acid, N-methyl-D-aspartate (NMDA), 1-oleoyl-2-acetyl-sn-glycerol (OAG), L-phenylephrine HCl, quisqualic acid, scopolamine HBr, tetrodotoxin (TTX), (Sigma); spiperone, ritanserin, (gift from Janssen); (\pm)-8-hydroxy-dipropylaminotetralin HBr (8-OH-DPAT), (\pm)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl (DOI), m-trifluoromethylphenylpiperazine HCl (TFMPP), (Research Biochemicals Inc.); prazosin HCl (gift from Pfizer); methysergide, ICS 205-930 (gift from Sandoz).

2.3 Results

2.3.1 Effects of 5-HT on NMDA depolarization

Bath application of 5-HT (10 or 30 μ M; 2 to 5 min, n=5) did not alter the recorded potential (c.f. Mally *et al.*, 1991). Nonetheless, in keeping with previous findings (Mally *et al.*, 1991), co-application of 5-HT (10 μ M) and NMDA (50 μ M) for 2 min resulted in a depolarization significantly larger in amplitude than the NMDA control (Fig. 4). The response to NMDA returned to control level following wash. All effects of 5-HT were reversible within 20-40 min. Since 5-HT did not induce a potential on its own, all non-EAA agonists were co-applied with an EAA agonist, unless stated otherwise.

The dose-response relationship for NMDA using a fixed concentration of 5-HT (30 μ M) is shown in Fig. 5A. The magnitude of facilitation was concentration dependent with maximum enhancement occurring at 50 μ M NMDA. This dose of NMDA yielded a submaximal depolarization which was reproducible for 10-12 hrs when NMDA was applied every 20 min. It was impossible to obtain a maximum for the NMDA dose-response curve, since responses with 100 μ M NMDA or greater did not recover completely (Harrison and Simmonds, 1985). Accordingly, 50 μ M NMDA was used for all experiments unless noted otherwise.

Construction of a dose response curve for 5-HT using repeated drug

applications in the same wedge proved unreliable due to prolonged desensitization following a single exposure (c.f. Araneda and Andrade, 1991; see Chapter 3). Only one concentration of 5-HT was therefore tested with each wedge. The concentration-response curve so constructed was not monotonic, i.e. the facilitation steadily increased from 1 to 30 μ M 5-HT, but was reduced at 100 μ M (Fig. 5B). The smaller response observed with 100 μ M 5-HT reflects multiple mechanisms of desensitization, which can be prevented with appropriate pharmacological agents (see Chapter 3).

While most of the wedges used were from young adult rats, a few wedges from postnatal (P-7) rats were tested in order to determine the 5-HT facilitation. Receptors for 5-HT appear early in development as judged by the presence of receptor mRNA (Roth *et al.*, 1991). Although not studied in detail, facilitation of the NMDA depolarization by 5-HT was present at postnatal day 5 (earlier dates not tested).

2.3.2 5-HT and EAA agonists

In cortical slices prepared from cat, but not rat, 5-HT facilitates the quisqualate induced depolarization (Nedergaard *et al.*, 1987; Reynolds *et al.*, 1988). Quisqualate and kainate were therefore perfused to determine the generality of the

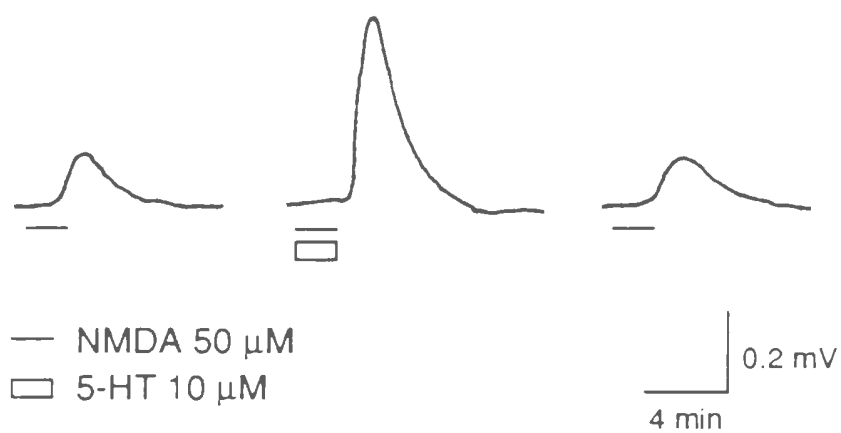
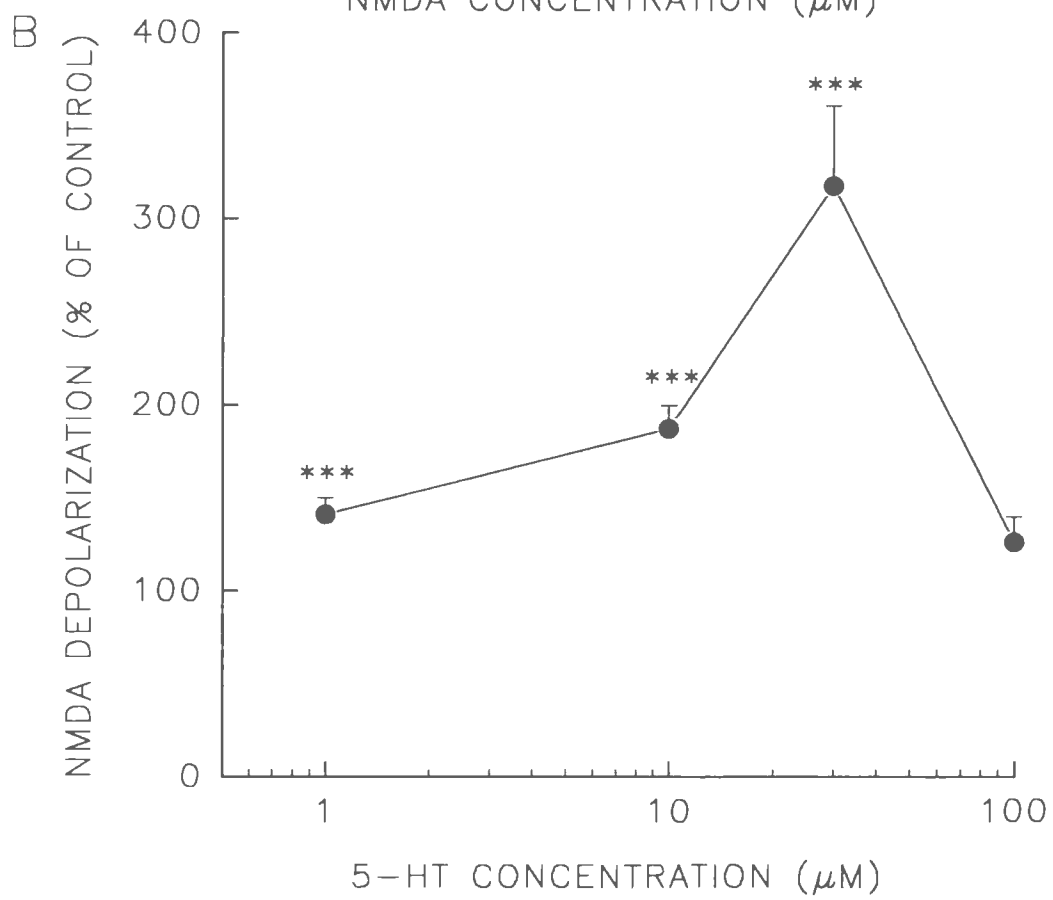
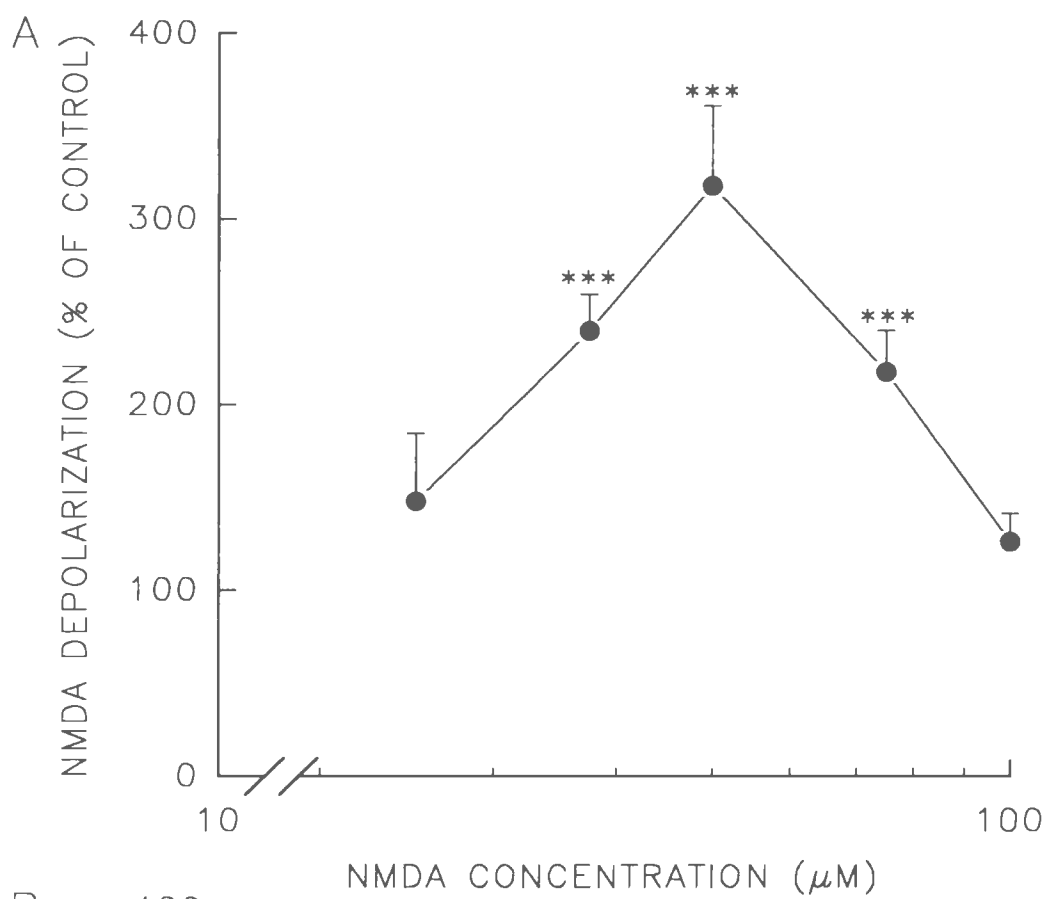


Figure 4. Facilitation of NMDA depolarization by 5-HT. NMDA depolarizes cortical neurons with respect to corpus callosum (left). Co-administration of 5-HT increases the amplitude of the NMDA response (centre). Recovery 20 min later (right). Upward deflection is negative.

Figure 5. Magnitude of the 5-HT facilitation dose-dependently varies with NMDA and 5-HT concentration. **A:** Concentration-response curve for NMDA facilitation. Concentration of 5-HT was 30 μ M throughout. Only one concentration of NMDA and 5-HT was tested on a wedge. 6 to 8 wedges were tested at each dose. **B:** Concentration-response relationship for 5-HT facilitation of NMDA depolarization. NMDA concentration was 50 μ M. 5 to 15 wedges were used for each point. ***, $p < 0.001$, treatment vs. control.



facilitation with respect to EAA receptor subtype. The response to each amino acid applied alone was similar to that described previously (Harrison and Simmonds, 1985). Co-application of 10 μ M 5-HT with submaximal concentrations of quisqualate and kainate (5 and 10 μ M, respectively) did not facilitate the response to either amino acid ($102 \pm 3\%$, $n=9$ and $104 \pm 4\%$, $n=6$ respectively; Fig. 6).

2.3.3 5-HT receptor antagonists

The receptor subtype mediating the NMDA facilitation was examined by co-applying 5-HT and NMDA in the presence of 5-HT receptor antagonists. To circumvent desensitization, each wedge was only exposed once to 30 μ M 5-HT plus antagonist. As shown in Fig. 7, ritanserin and spiperone reduced 5-HT facilitation of the NMDA depolarization in a concentration dependent manner. The IC_{50} values for ritanserin and spiperone were 0.9 and 0.4 nM, respectively. Methysergide, 1 and 10 nM, also significantly reduced the facilitation ($338 \pm 43\%$, $n=8$) induced by 5-HT ($204 \pm 16\%$, $n=6$, $p < 0.05$ and $174 \pm 8\%$, $n=6$, $p < 0.01$, respectively). The 5-HT facilitation was not reduced by ICS 205-930, a 5-HT₃ antagonist (Fig. 7). However, ICS 205-930 significantly reduced the NMDA depolarization to $72 \pm 6\%$ ($n=6$; $p < 0.05$) of the control. This was in sharp contrast to methysergide, ritanserin and spiperone which did not reduce the NMDA response.

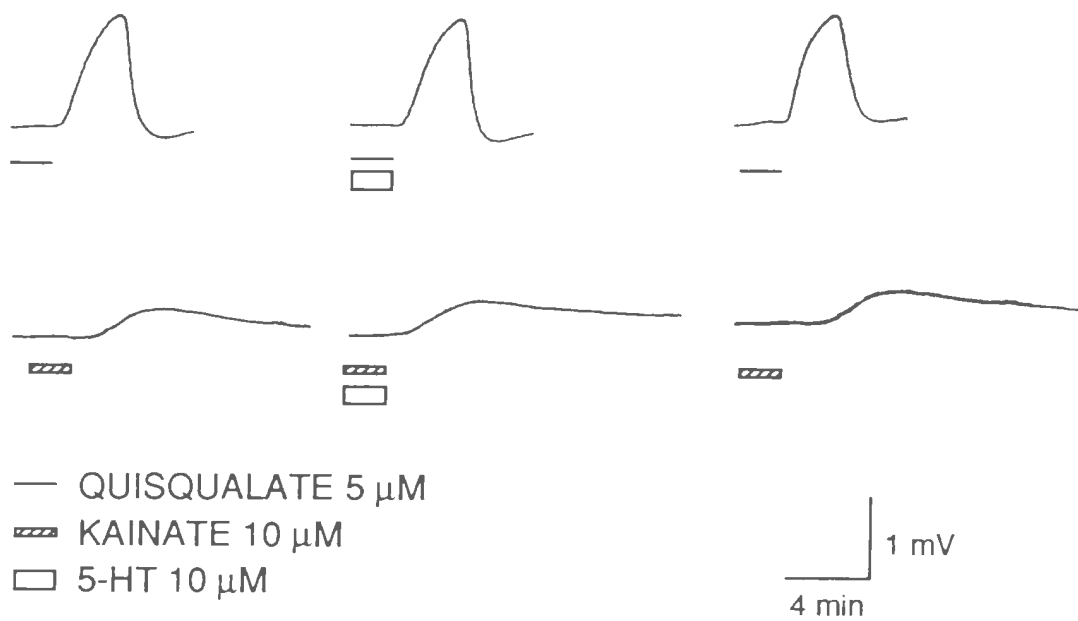


Figure 6. Depolarization of cortical neurons by quisqualate and kainate is not enhanced by 5-HT co-administration. Control responses are at left, recovery 20 min later at right.

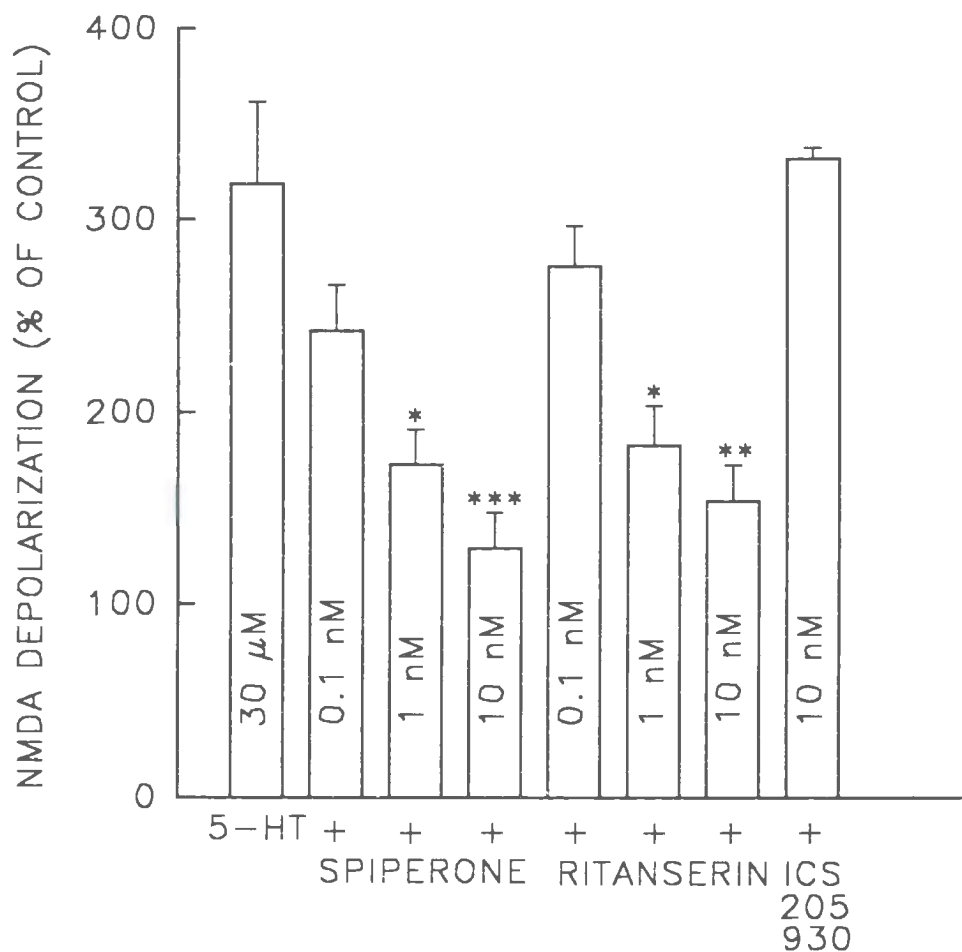


Figure 7. 5-HT_{2A} receptor antagonists dose-dependently reduce 5-HT facilitation. Antagonists were bath applied 30-40 min before 5-HT was tested. NMDA depolarization in the presence of antagonist was used as control. 6 to 8 wedges were used at each antagonist concentration. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ (Bonferroni p values adjusted for multiple comparisons).

2.3.4 5-HT receptor agonists

DOI, TFMPP, and 8-OH-DPAT were substituted for 5-HT to examine effects of additional 5-HT receptor agonists on the NMDA depolarization (Fig. 8). DOI, a mixed 5-HT_{2A} and 5-HT_{2C} receptor agonist significantly and reversibly enhanced the NMDA response at 2 and 5 μ M. However, at 10 μ M DOI, there was no facilitation ($85 \pm 7\%$, $n=5$, not significant). TFMPP (5 μ M, mixed 5-HT_{1B} and 5-HT_{2C} agonist) did not significantly alter the response to NMDA. In these wedges, DOI (5 μ M), applied after washing TFMPP, resulted in facilitation ($240 \pm 14\%$, $n=4$). 8-OH-DPAT (10 μ M; 5-HT_{1A} agonist) significantly reduced the amplitude of the NMDA depolarization ($59 \pm 9\%$, $n=6$, $p < 0.01$). This inhibition by 8-OH-DPAT persisted in the presence of TTX (0.5 μ M) and was completely antagonized by spiperone (1 μ M; $n=6$; not shown).

2.3.5 Cholinergic and adrenergic agonists and antagonists

Like 5-HT, muscarinic cholinergic and α_1 -adrenoceptor agonists facilitate NMDA and glutamate evoked activity on cortical neurons (Metherate *et al.*, 1987; Mouradian *et al.*, 1991). Moreover, release of acetylcholine and norepinephrine might alter the response to 5-HT in the neocortex (Hansson *et al.*, 1990). Perfusion of carbachol (10 μ M) and phenylephrine (10 μ M) enhanced the NMDA evoked depolarization ($178 \pm 9\%$, $n=6$, $p < 0.01$; and $155 \pm 7\%$, $n=6$, $p < 0.001$,

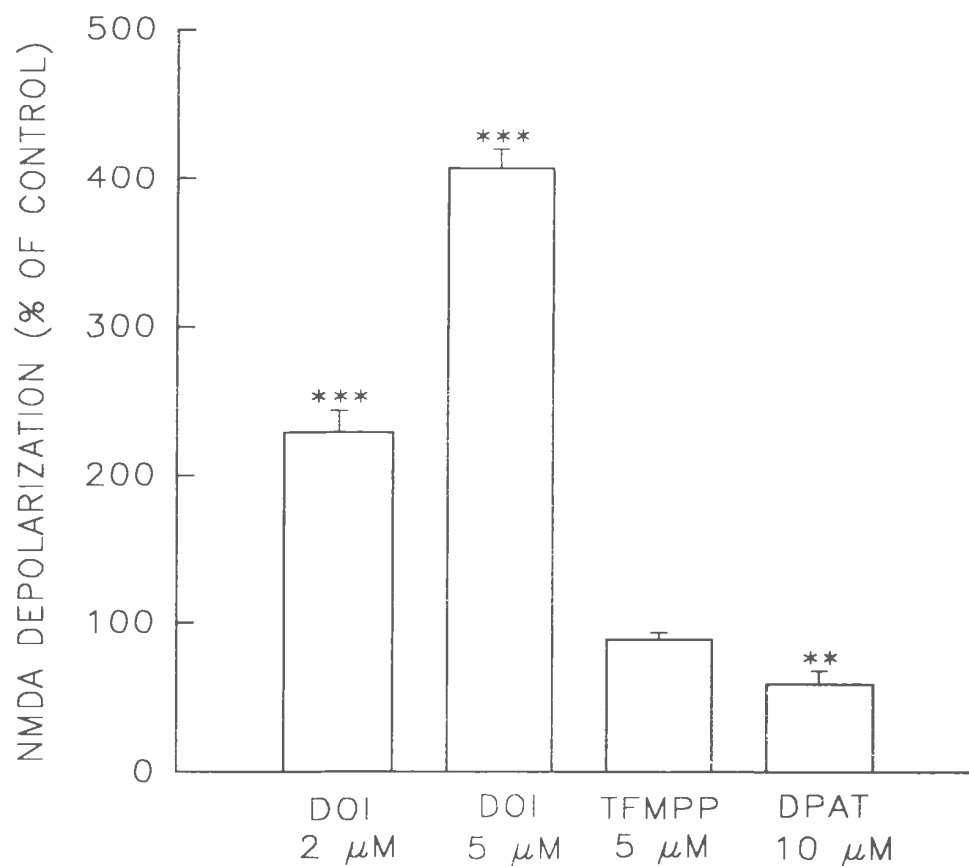


Figure 8. Effects of 5-HT receptor agonists on NMDA depolarization. Only DOI enhances the NMDA response. DPAT (8-OH-DPAT). **, $p < 0.01$, ***, $p < 0.001$ (agonist plus NMDA vs NMDA).

respectively), but neither agonist depolarized cortical neurons in the absence of NMDA (c.f. Harrison and Simmonds, 1985). Facilitation observed with these agonists was blocked by scopolamine and prazosin respectively (data not shown). Interestingly, scopolamine and prazosin significantly reduced the NMDA depolarization (Fig. 9). However, neither antagonist significantly reduced the facilitation induced by 5-HT 30 μ M (Fig. 9).

2.3.6 5-HT facilitation in tetrodotoxin

The contribution of ongoing electrical activity to the 5-HT facilitation was evaluated by blocking fast Na⁺ currents with 0.3 μ M TTX. In contrast to previous reports (Harrison and Simmonds, 1985; Burton *et al.*, 1987), TTX consistently reduced the response to NMDA (Fig. 10). Despite this reduction, the facilitation induced by 10 and 30 μ M 5-HT persisted (Fig. 10).

2.3.7 5-HT facilitation in barium

There is general agreement that the 5-HT_{2A} receptor mediated depolarization results from a closure of K⁺ channels (see Anwyl, 1990 for review). In the nucleus accumbens, the depolarization resulting from activation of 5-HT_{2A} receptors is occluded by barium (North and Uchimura, 1989). In wedges perfused with 100 μ M barium, the NMDA depolarization was reduced ($52 \pm 18\%$, $n=6$, $p < 0.05$), but

the facilitation with 30 μ M 5-HT persisted ($230 \pm 38\%$, $n=6$, $p < 0.01$). Clearly, the NMDA facilitation is not dependent on the 5-HT_{2A} mediated depolarization.

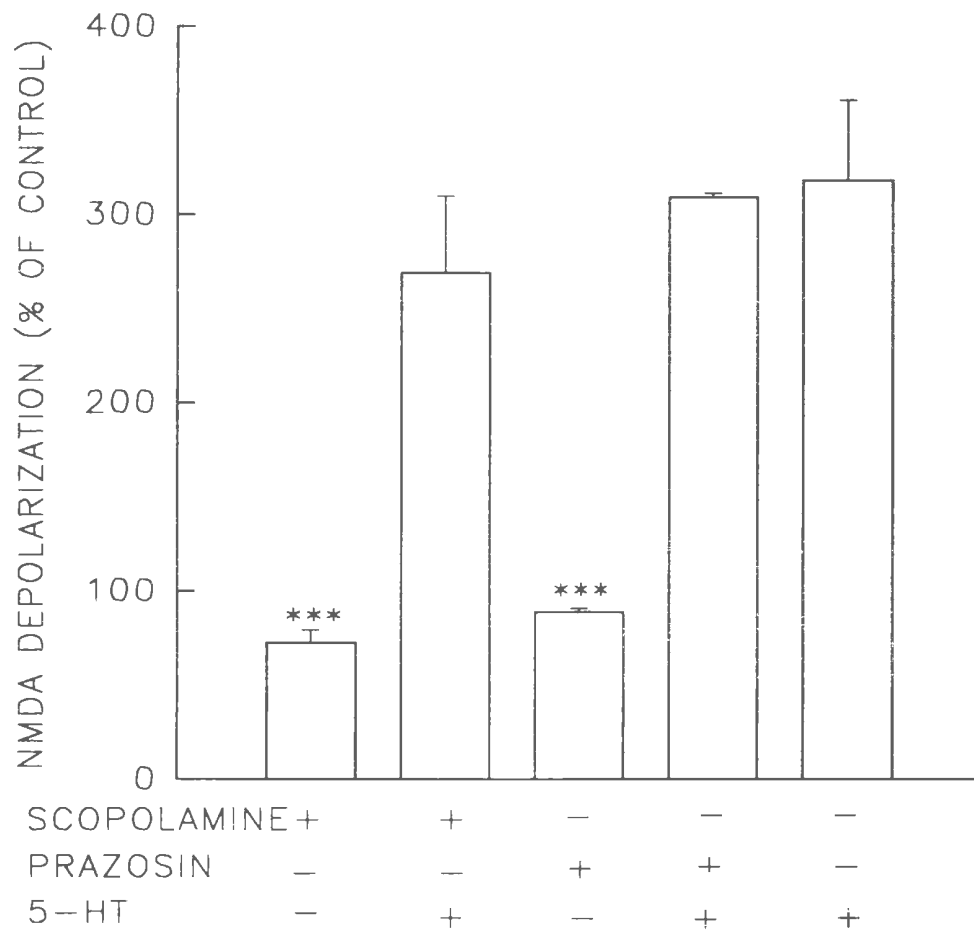


Figure 9. Muscarinic cholinergic and α_1 -adrenoceptor antagonists reduce the NMDA response, but not 5-HT facilitation. Scopolamine (10 nM; n=6) and prazosin (1 μ M; n=6) significantly reduce the NMDA response (NMDA vs NMDA plus antagonist, paired t test). When co-applied with 30 μ M 5-HT, neither antagonist significantly reduces the facilitation. ***, $p < 0.001$.

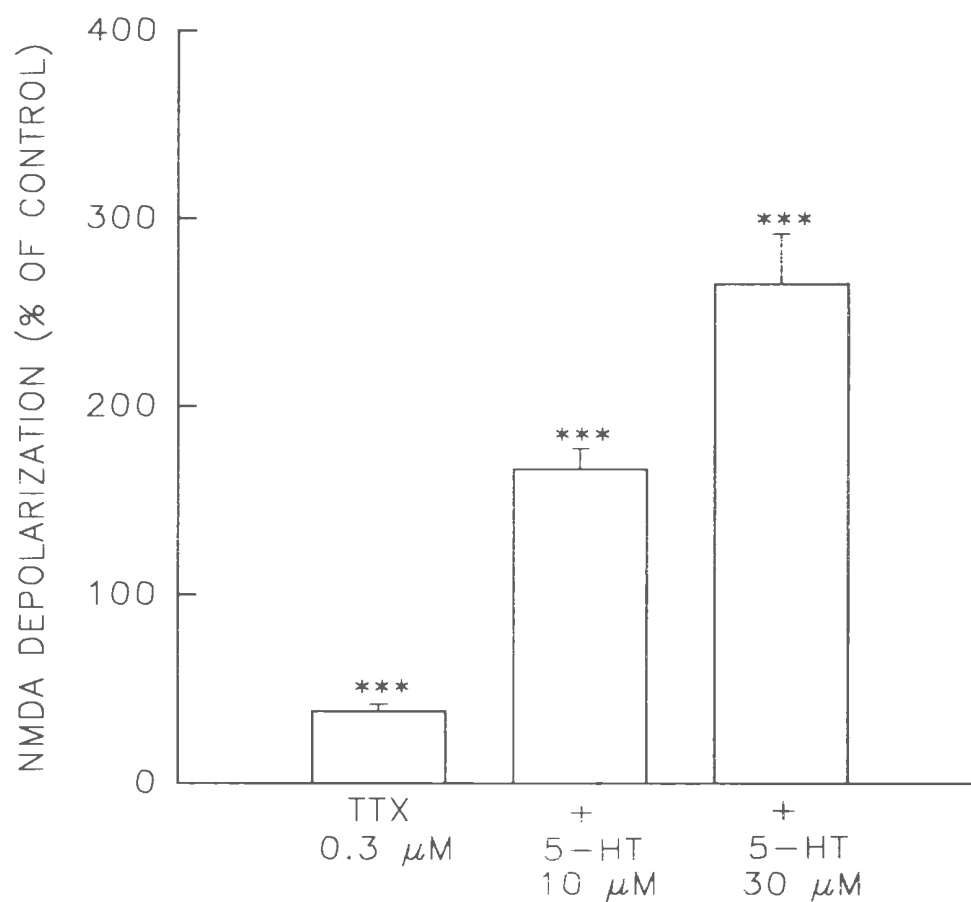


Figure 10. Effect of TTX on NMDA depolarization and facilitation by 5-HT. TTX (0.3 μ M) significantly reduces the NMDA depolarization (NMDA vs NMDA and TTX; paired t test). However, the facilitation by 5-HT persists (NMDA and TTX vs 5-HT, NMDA plus TTX; Bonferroni test). **, $p < 0.01$, ***, $p < 0.001$.

2.4 Discussion

2.4.1 5-HT receptor subtype mediating NMDA facilitation

Numerous 5-HT receptor subtypes are present in rat cerebral cortex. Those so far identified include 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C}, 5-HT₃, 5-HT₄, 5-HT₅, 5-HT₆ and 5-HT₇ receptors (Kilpatrick *et al.*, 1987; Edwards *et al.*, 1991; see Palacios and Dietl, 1988; Zifa and Fillion, 1992; Hoyer *et al.*, 1994 for review). Of these receptors, the present findings are only compatible with activation of 5-HT_{2A} receptors leading to facilitation of the NMDA depolarization. Methysergide, ritanserin and spiperone concentration-dependently reduce the response to 5-HT with subnanomolar concentrations reducing the facilitation by 50%. Although methysergide binds to several 5-HT sites, it is the 5-HT_{2A} and 5-HT_{2C} sites for which it demonstrates the highest affinity (Peroutka, 1988). These two receptors have considerable sequence homology, which explains the close affinity for many antagonists (Julius *et al.*, 1990). Ritanserin exhibits greater selectivity than does methysergide, significant binding occurring only at 5-HT_{2A} and 5-HT_{2C} sites (Leysen *et al.*, 1985). Ritanserin also binds to dopamine-D₂, α_1 -adrenoceptor, α_2 -adrenoceptor, and histamine (H₁) sites, but with lower affinity than at 5-HT sites (Leysen *et al.*, 1985). Accordingly, ritanserin is not likely to result in significant antagonism at these receptors with the low concentrations employed. Spiperone,

in contrast to methysergide and ritanserin, clearly differentiates between 5-HT_{2A} and 5-HT_{2C} receptors in both functional testing (K_i of 4.3 nM and 6200 nM, respectively for inhibition of 5-HT stimulated phosphoinositide hydrolysis; Conn *et al.*, 1986) and in binding assays (Peroutka, 1988). In light of the results with methysergide and ritanserin, implicating a 5-HT receptor, and the effectiveness of spiperone in antagonizing the 5-HT facilitation, it is concluded that 5-HT_{2A} receptors mediate the enhancement of the NMDA response observed in these studies.

DOI was the only 5-HT receptor agonist to mimic the 5-HT enhancement. DOI labels high affinity (guanyl nucleotide sensitive) 5-HT_{2A} sites located in rat neocortex (Appel *et al.*, 1990). In functional studies, DOI stimulates phosphoinositide hydrolysis in both choroid plexus and neocortex through activation of 5-HT_{2A} and 5-HT_{2C} receptors respectively (Conn and Sanders-Bush, 1987). DOI is a partial agonist (Kagaya *et al.*, 1990) and consonant with that property, the maximum facilitation observed with DOI is smaller than that found with 5-HT, despite the latter's susceptibility to oxidation and uptake. As with 5-HT, desensitization develops at elevated DOI concentrations (see Chapter 3). The NMDA response is reduced by 8-OH-DPAT, excluding consideration of 5-HT_{1A} receptors in mediating the facilitation. TFMPP is an agonist at 5-HT_{1B} and 5-HT_{2C} receptors (Sills *et al.*, 1984; Conn and Sanders-Bush, 1987), but an antagonist at

cortical 5-HT_{2A} receptors (Conn and Sanders-Bush, 1987; Neuman and Zebrowska, 1992). The failure of TFMPP to enhance the NMDA depolarization lends further support to the proposition that 5-HT_{2C} receptors do not mediate the facilitation.

It should be noted, the present finding that 5-HT_{2A}, but not 5-HT₁, receptor activation mediates the NMDA enhancement differs from that of Mally *et al.* (1991). They report (\pm) propranolol blocks 5-HT enhancement of the NMDA depolarization in mouse cortical wedges and suggest 5-HT₁ receptors mediate the response. However, propranolol is an antagonist at 5-HT_{2A} receptors (Hoyer *et al.*, 1994) and at the concentration employed (40 μ M) in their study, this could explain their observation.

The presence of inhibitory 5-HT_{1A} receptors likely accounts for the greater than expected potency of 5-HT_{2A} antagonists observed in the present study (c.f. North and Uchimura, 1989). The present study suggests that application of 5-HT stimulates both 5-HT_{1A} and 5-HT_{2A} receptors and the resulting response, therefore, represents a balance between inhibition and excitation. In the presence of 5-HT_{2A} antagonists the inhibitory 5-HT_{1A} action is unmasked. Lakoski and Aghajanian (1985) observed a similar phenomenon *in vivo* with iontophoretic application of ketanserin. *In vitro* studies reveal both 5-HT_{2A} and 5-HT_{1A} receptors are found on the same layer V neurons (Davies *et al.*, 1987; Araneda and Andrade, 1991) which supports this hypothesis. Carbachol and phenylephrine stimulate phosphoinositide

hydrolysis in brain slices as does activation of 5-HT_{2A} receptors (Brown *et al.*, 1984; Nahorski, 1988), so it may not be surprising they exhibit comparable cellular effects including facilitation of NMDA depolarization. This commonality raises the question of whether 5-HT acts indirectly to release acetylcholine or norepinephrine, thereby enhancing the NMDA response. Moreover, lesioning forebrain cholinergic neurons reveals some 5-HT_{2A} binding sites are situated on cholinergic terminals (Palacios and Dietl, 1988). Nonetheless, such indirect action is doubtful because scopolamine and prazosin do not significantly reduce the 5-HT facilitation.

2.4.2 Neurons contributing to the response

Previously, neither binding nor *in situ* hybridization studies provided sufficient resolution to unequivocally localize 5-HT_{2A} receptors to interneurons or pyramidal (projection) neurons (Mengod *et al.*, 1990; Pompeiano *et al.*, 1994). However, it is clear that 5-HT_{2A} receptors are expressed by pyramidal neurons (see Chapter 6). Findings from intracellular recording studies on layer V neurons are so far consistent with a postsynaptic localization for 5-HT_{2A} receptors (Davies *et al.*, 1987; Araneda and Andrade, 1991). Persistence of the 5-HT facilitation in TTX accords well with intracellular studies (Nedergaard *et al.*, 1986, 1987; Reynolds *et al.*, 1988). The known excitatory effects of 5-HT_{2A} receptor

stimulation (see Anwyl, 1990, for review) are not easily reconciled with a disinhibitory mechanism. Thus, in the absence of evidence for an indirect effect, it is likely that 5-HT_{2A} receptors mediating the facilitation are situated on projection neurons (see Chapter 6). Moreover, these neurons are most likely layer V pyramidal neurons since: (i) layer V neurons form the largest group of projection neurons (Lorente De No, 1949), thus likely contributing substantially to the NMDA depolarization; and (ii) this region contains the highest concentration of 5-HT_{2A} binding sites (see Chapter 1).

Other neurons, including interneurons and nerve fibres (e.g. monoamine containing) in the vicinity of projection neurons, doubtless influence the NMDA depolarization through release of neurotransmitter or neuromodulatory substances. The significant reduction of the NMDA response observed with TTX, scopolamine, prazosin, and ICS 205-930 lends credence to this view. Liberation of cholinergic and adrenergic agonists is implied by the present findings with scopolamine and prazosin. Agonists may be released tonically, but more likely, are evoked by NMDA application (Lalies *et al.*, 1988). Conceivably, TTX and ICS 205-930 reduce the NMDA response by decreasing the release of acetylcholine, norepinephrine or other agonists (see below). Moreover, the effectiveness of 10 nM ICS 205-930 in reducing the NMDA response suggests some form of 5-HT release is taking place. Liberated 5-HT does not directly enhance the NMDA

response through stimulating 5-HT_{2A} receptors though, since methysergide, ritanserin and spiperone do not depress the NMDA depolarization.

2.4.3 Tetrodotoxin sensitivity

The TTX sensitive nature of the NMDA depolarization has not previously been reported in comparable studies on rat and mouse cortical wedges (Harrison and Simmonds, 1985; Burton *et al.*, 1987). A plausible explanation for this discrepancy is the use of Mg⁺⁺-free ACSF (Harrison and Simmonds, 1985; Burton *et al.*, 1987), in contrast to Mg⁺⁺ containing ACSF in the present study. Enhancement of the NMDA depolarization by 5-HT and phenylephrine is eliminated following 1-2 hr wash in Mg⁺⁺-free ACSF (Chapter 5). Similarly, muscarinic depolarization of cortical neurons is abolished in Mg⁺⁺-free media (El-Beheiry and Puil, 1990). Consequently, in Mg⁺⁺-free media, agonists which would normally confer TTX sensitivity to the NMDA depolarization are inactive.

Thus, it is clear that activation of 5-HT_{2A} receptors facilitates the NMDA response of neocortical projection neurons. However, the mechanisms responsible for the facilitation remain to be investigated. As one possibility, the desensitization of the 5-HT_{2A} receptor mediated response observed in this study could yield potential clues in understanding the mechanisms that underlie the facilitation. The following chapter deals with this issue.

CHAPTER 3

MULTIPLE MECHANISMS OF 5-HT_{2A} RECEPTOR DESENSITIZATION

3.1 Introduction

While characterizing enhancement of the NMDA depolarization of cortical neurons by 5-HT, it was observed that the 5-HT facilitation exhibited desensitization (Chapter 2). Desensitization is a widespread phenomenon in which initial exposure to an agonist results in decreased cellular responsiveness on second exposure. 5-HT receptors, like many other receptors, are susceptible to agonist induced desensitization. Thus, 5-HT_{2A} receptors located on cortical pyramidal neurons (Araneda and Andrade, 1991; Leysen *et al.*, 1989), cerebellar granule cells (Dillon-Carter and Chuang, 1989), facial motoneurons (Aghajanian, 1990) and platelets (Kagaya *et al.*, 1990) undergo desensitization. Not with other receptors leading to activation of protein kinase C, desensitization of 5-HT_{2A} receptors results from feedback inhibition mediated by protein kinase C (Aghajanian, 1990; Roth *et al.*, 1986). Such inhibition may serve i) to protect cells from over stimulation, ii) as a modulatory substrate for other regulators of cellular activity.

A second mechanism leading to loss of receptor mediated responsiveness is internalization or sequestration of receptors by which hydrophilic agonists are

prevented access to receptors. Sequestration of β -adrenoceptors has been extensively studied (Waldo *et al.*, 1983; Towes *et al.*, 1984; Kassir *et al.*, 1986). Sequestration of β -adrenoceptors occurs in the presence of high concentrations of agonist (Waldo *et al.*, 1983). Sequestration of 5-HT₄ receptors has been recently demonstrated (Ansanay *et al.*, 1992), but sequestration of 5-HT_{2A} receptors has not been reported (see Roth *et al.*, 1990 for review).

In light of the above studies and observations and to better understand the phenomenon, the present study was undertaken to examine the mechanisms underlying desensitization of cortical 5-HT_{2A} receptors.

3.2 Methods

3.2.1 Wedge preparation and recording

Wedges from rat sensorimotor cortex were prepared for recording and analyzed as described in section 2.2.2 and 2.2.5.

3.2.2 ACSF composition and drug application

ACSF composition in the bathing medium and drug application were as described in section 2.2.3 and 2.2.4 with the exception of the following drugs: phorbol diacetate, oleoacetyl glycerol, staurosporine and calmidazolium were dissolved in dimethylsulfoxide (100%) and diluted in ACSF. Dimethylsulfoxide (0.05%, vol/vol, the largest concentration following dilution) alone did not alter the NMDA depolarization. Phorbol diacetate and oleoacetyl glycerol were perfused for 20 min whereas staurosporine, calmidazolium, chlorpromazine, amiloride and concanavalin A were perfused for 40 min before addition of agonists.

3.2.3 Drugs and chemicals

The following drugs were used: calmidazolium, concanavalin A, 1-oleoyl-2-acetyl-n-glycerol (oleoacetyl glycerol), 4 β -phorbol 12,13-diacetate (phorbol diacetate), and staurosporine were obtained from Sigma; amiloride HCl (gift from

Merck Sharp & Dohme Research Lab.); chlorpromazine HCl (gift from Rhone-Poulenc Pharma Inc).

3.3 Results

3.3.1 Desensitization of 5-HT response

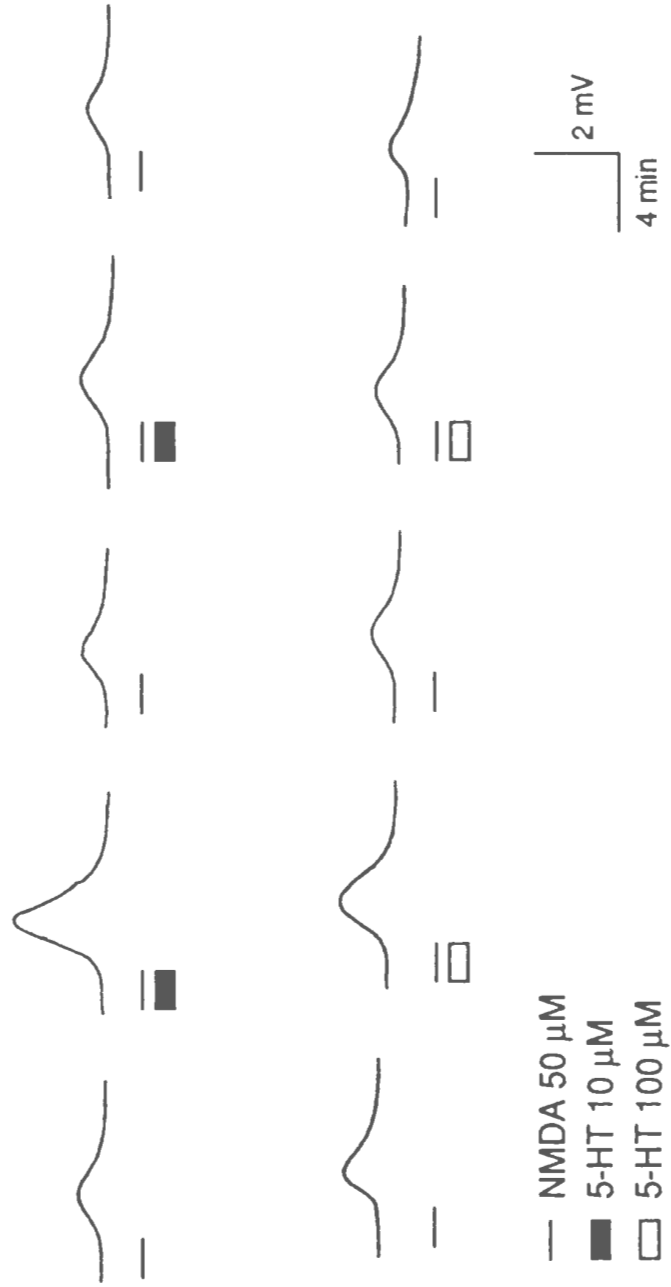
In keeping with previous observations (Chapter 2), co-administration of 5-HT (10 μ M) and NMDA reversibly enhanced the NMDA depolarization of rat cortical neurons by $229 \pm 8\%$ (n=18). However, on second exposure 40 min later, this was reduced to $151 \pm 6\%$, n=6; (Fig. 11, upper panel) thus demonstrating the presence of desensitization. Qualitatively similar results were obtained when the concentration of 5-HT was raised to 100 μ M, but with the additional feature that the initial enhancement was smaller in amplitude than that observed with 10 μ M 5-HT (Fig. 12, lower panel).

Time course of recovery from desensitization following initial exposure to 10 μ M 5-HT is shown in Fig. 12. Each cortical wedge was exposed to 5-HT twice, the second application occurring 40, 80 or 120 min following the first. Only at 120 min did the 5-HT response return to the control level. In contrast to the 5-HT facilitation, the depolarization evoked by NMDA alone did not exhibit desensitization (Fig. 12).

3.3.2 Staurosporine reduces desensitization

To evaluate the contribution of protein kinase C to the observed

Figure 11. Facilitation of NMDA depolarization by 5-HT undergoes desensitization. Upper panel: NMDA induced depolarization is facilitated by 10 μ M 5-HT. On second exposure to 5-HT 40 min later, the magnitude of the facilitation is reduced. Note the depolarization evoked by NMDA alone is relatively unchanged. Lower panel: desensitization also takes place when 100 μ M 5-HT is employed. Note the magnitude of the facilitation is smaller than that observed with 10 μ M 5-HT.



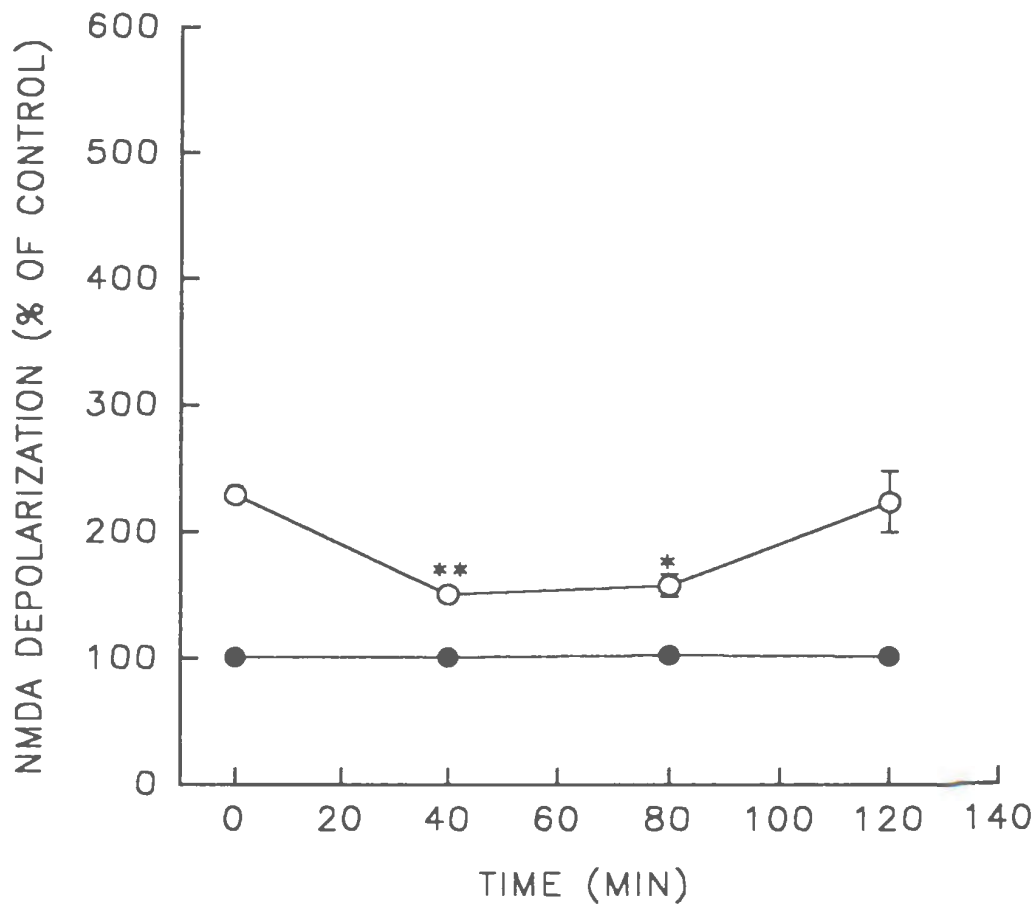


Figure 12. Time course of recovery from desensitization. Each wedge was exposed to 5-HT twice, separated by intervals of 40, 80, 120 min (○). Applying NMDA alone (●) does not result in desensitization. Error bars for the NMDA responses are smaller than the symbol. The 5-HT data are from 20 wedges at time 0, 10 wedges at 40 min and 5 wedges at each time point thereafter. NMDA data averaged from 10 wedges. *, $p < 0.05$, **, $p < 0.01$, 5-HT vs 5-HT at time 0.

desensitization, staurosporine, an inhibitor of protein kinase C (Tamaoki *et al.*, 1986; Davis *et al.*, 1992) was employed. Perfusion of staurosporine (10 nM) was followed by a test concentration of 5-HT. After a further 40 min the same concentration of 5-HT was reapplied. In this manner, a concentration response curve was generated for 5-HT in the presence of staurosporine and compared with untreated wedges (Fig. 13). On first exposure to 5-HT, staurosporine significantly potentiated 5-HT enhancement of the NMDA depolarization at all 5-HT concentrations except 30 μ M (Fig. 13A). While the potency and efficacy of 5-HT was enhanced, staurosporine did not eliminate the apparent loss of facilitation in efficacy at elevated 5-HT concentrations. Moreover, the inflection point for the decline was shifted to the left (Fig. 13A). Staurosporine alone did not alter the NMDA depolarization.

Facilitation of the NMDA depolarization (Chapter 2) and desensitization were also observed when DOI (5 μ M) was substituted for 5-HT (Table II). As with 5-HT, pretreatment with staurosporine potentiated DOI enhancement of the NMDA depolarization. However, in the case of DOI, staurosporine entirely prevented the desensitization observed on second exposure (Table II).

3.3.3 Protein kinase C activators prevent 5-HT facilitation

If inhibition of protein kinase C potentiates the 5-HT facilitation, activators

Figure 13. Bath perfusion with staurosporine potentiates 5-HT facilitation. **A.** On first exposure to 5-HT in the presence of staurosporine (○; ST) the facilitation is potentiated compared to the response in control wedges (●). Note, staurosporine shifts both the maximal facilitation and the inflection point for decline of facilitation to the left. **B.** On second exposure to 5-HT, staurosporine continues to potentiate the 5-HT facilitation. The potentiated response is smaller at each dose compared to A, but the difference does not reach significance. 5 to 18 wedges were used at each concentration of 5-HT. *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$; 5-HT plus staurosporine vs. 5-HT.

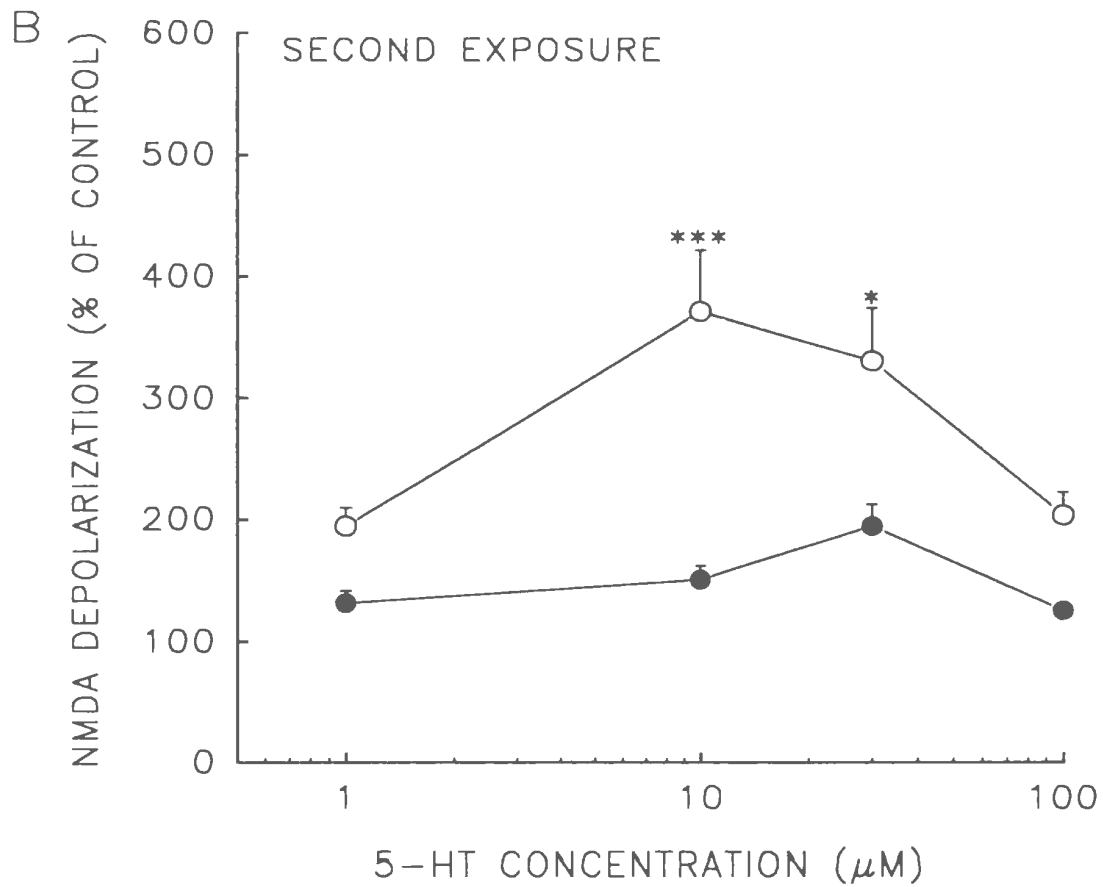
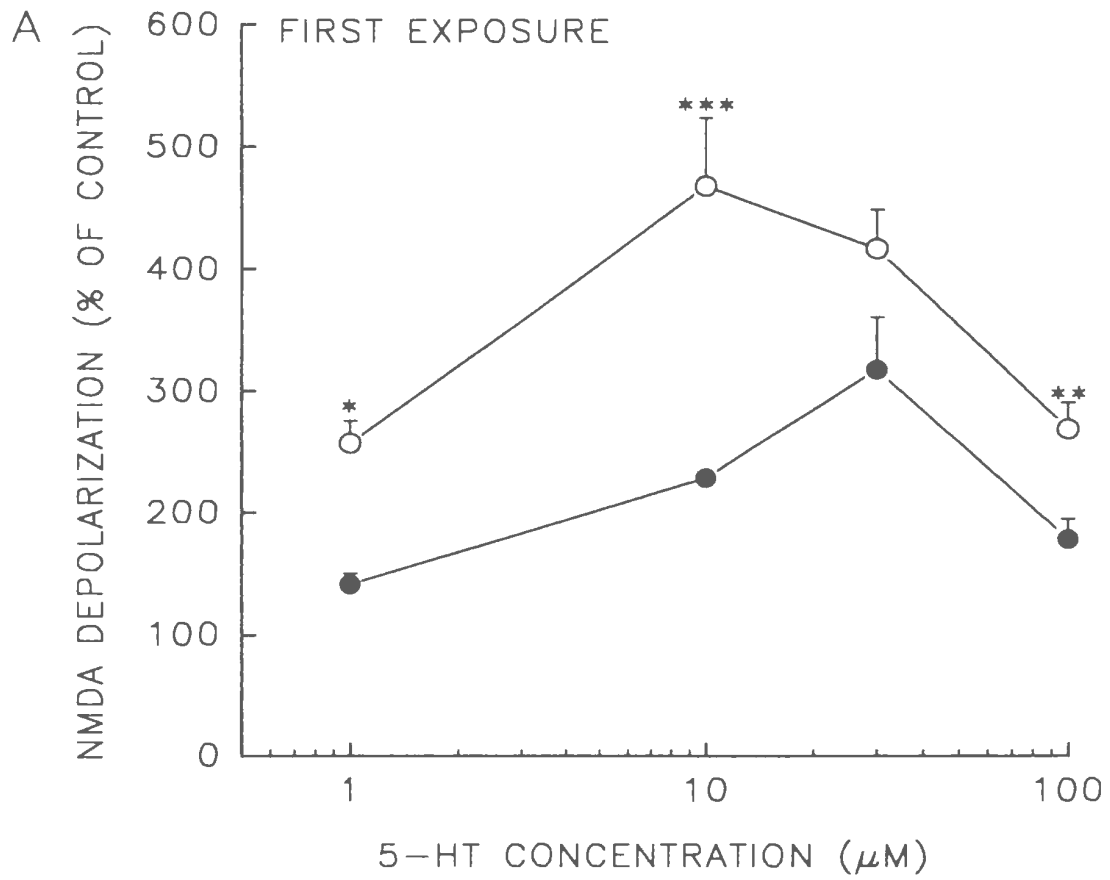


Table II. Potentiation of DOI facilitation by staurosporine

Drug	NMDA depolarization (% of Control)	
	First exposure	Second exposure
DOI 5 μ M	(n=6) 263 \pm 10%	178 \pm 16% ^a
DOI 5 μ M		
+ Staurosporine	(n=6) 339 \pm 11% ^b	331 \pm 19% ^b

^a $p < 0.01$, DOI, first exposure vs. second exposure;

^b $p < 0.05$, DOI vs. DOI + Staurosporine.

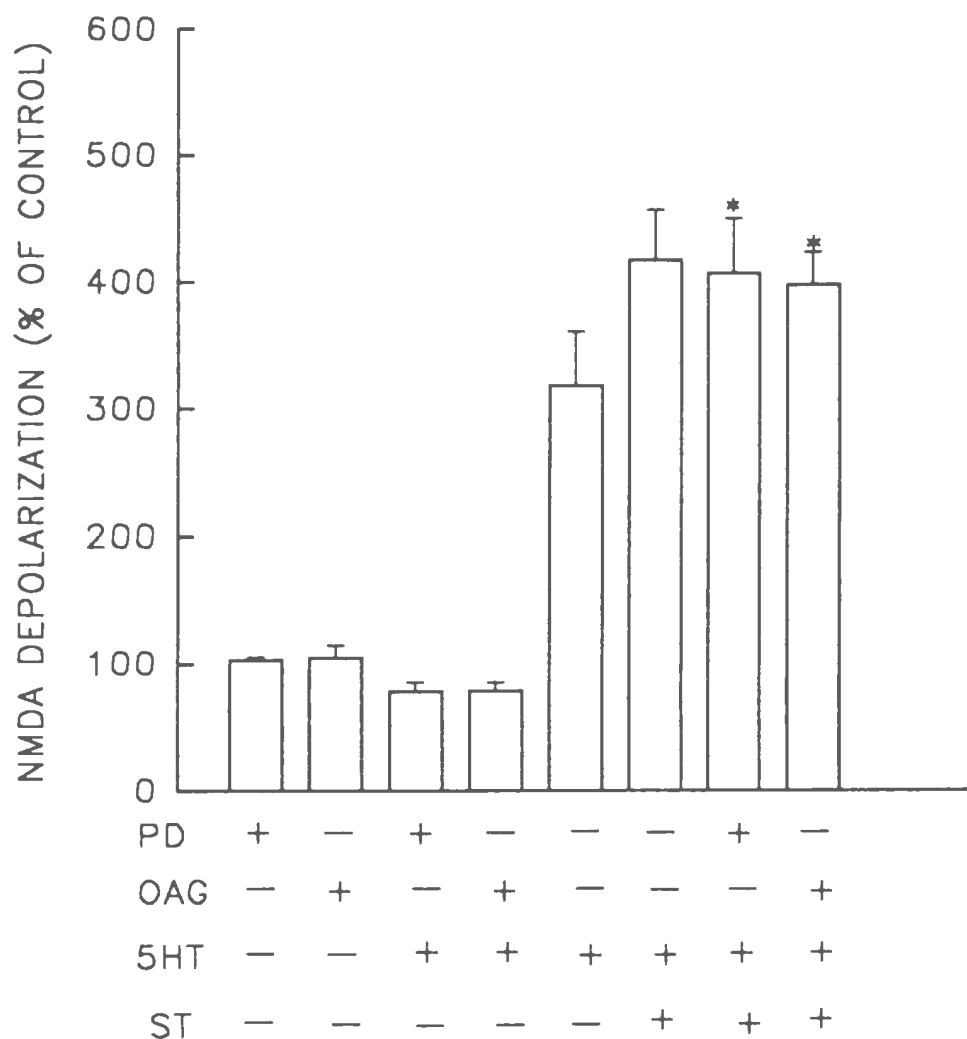


Figure 14. Activation of protein kinase C blocks the 5-HT facilitation. Bath perfusion with phorbol diacetate (PD; 3 μ M) or oleoacetyl glycerol (OAG; 50 μ M) did not alter the magnitude of the NMDA depolarization, whereas they both completely blocked the 5-HT facilitation. Staurosporine (ST) fully restored the facilitation. 6 to 8 wedges were used for each treatment. *, $p < 0.05$, phorbol diacetate and oleoacetyl glycerol with and without staurosporine.

of protein kinase C should reduce it. To test this prediction, protein kinase C was stimulated with phorbol diacetate (3 μ M) and oleoacetyl glycerol (50 μ M; Agopyan and Agopyan, 1991; Singer *et al.*, 1990; Lai *et al.*, 1990). Neither phorbol diacetate or oleoacetyl glycerol altered the NMDA depolarization (Fig. 14). However, both agents eliminated the facilitation observed with 5-HT 30 μ M, an effect that was prevented by prior treatment with staurosporine (10 nM; Fig. 14).

3.3.4 Concanavalin A and desensitization

Concanavalin A prevents sequestration of several receptor types (see section 3.4). Exposure to concanavalin A (100 nM) significantly potentiated the facilitation observed with 100 μ M 5-HT (Fig. 15), but not lower 5-HT concentrations (i.e. $165 \pm 19\%$, $n=4$; $289 \pm 10\%$, $n=4$ at 10 μ M and 30 μ M 5-HT respectively). The NMDA depolarization in the absence of 5-HT was not altered by concanavalin A ($103 \pm 5\%$; $n=5$). Concanavalin A reportedly increases intracellular pH through an amiloride sensitive mechanism (Mironov, 1992). A rise in intracellular pH might potentiate the 5-HT response by a mechanism other than preventing receptor sequestration (see section 3.4). As shown in Fig. 15, amiloride reduced the 5-HT facilitation in the presence of concanavalin A in a concentration-dependent manner. However, amiloride also blocked the 5-HT facilitation in the absence of concanavalin A (Fig. 15). Amiloride alone at 1 mM, but not 10 μ M,

significantly enhanced the NMDA depolarization ($150 \pm 17\%$, $n=5$; $p < 0.001$; Fig. 15).

3.3.5 Calmodulin antagonists and desensitization

Despite significant potentiation of the 5-HT (100 μ M) response observed with concanavalin A and staurosporine, wedges pretreated with both agents exhibited some desensitization (Fig. 16). Since activation of 5-HT_{2A} receptors increases intracellular Ca²⁺ (Berridge, 1987), a Ca²⁺-dependent binding protein such as calmodulin might participate in desensitization. This possibility was tested using the calmodulin antagonists, calmidazolium and chlorpromazine (Chuprun *et al.*, 1991; Masson *et al.*, 1992). As shown in Fig. 16, neither calmidazolium nor chlorpromazine significantly altered 5-HT (100 μ M) facilitation of the NMDA response; however, they fully prevented desensitization on second exposure to the agent. Moreover, when the 5-HT facilitation was potentiated with concanavalin A, calmidazolium remained effective in eliminating desensitization on second exposure (Fig. 16).

3.3.6 Heterologous desensitization

Carbachol and phenylephrine also facilitate NMDA receptor mediated depolarization of cortical neurons (Chapter 2). Like 5-HT, application of

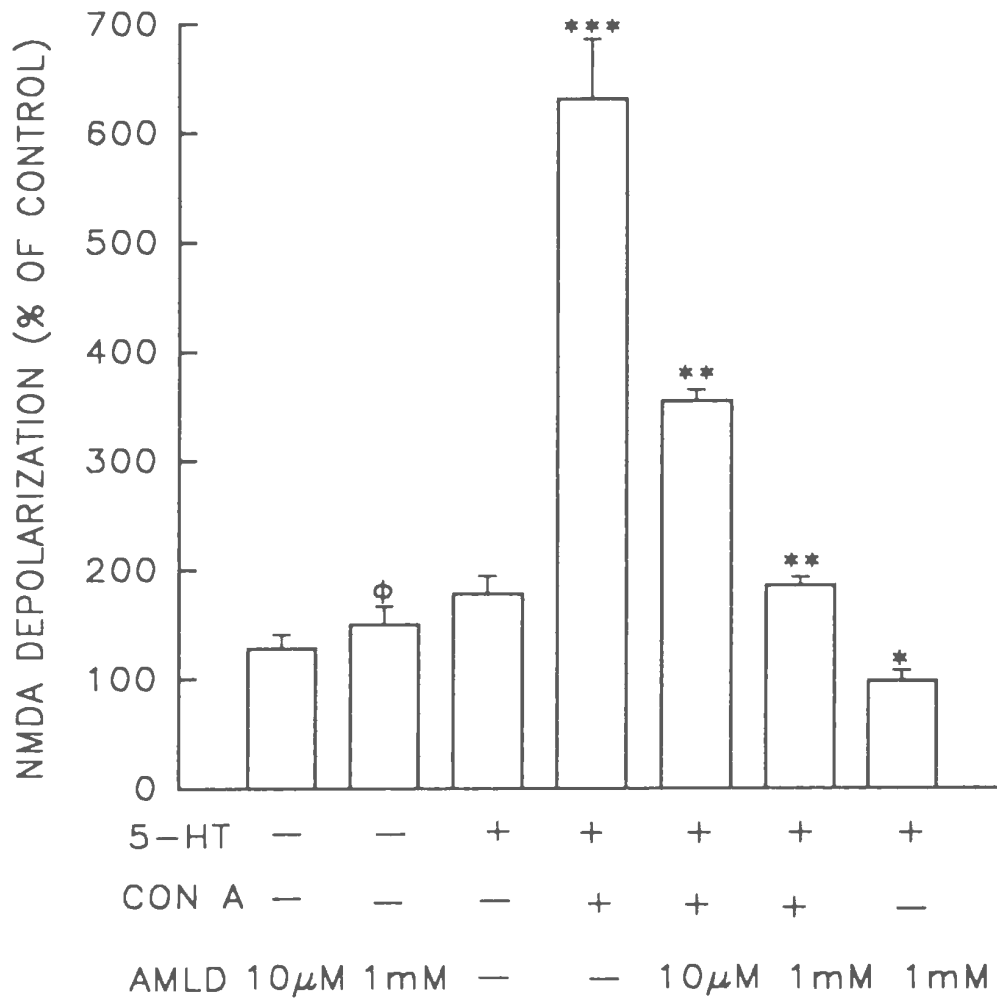


Figure 15. Concanavalin A (CON A; 100 nM) potentiates 5-HT (100 μM) facilitation of NMDA depolarization. Amiloride (AMLD) reduces the facilitation in a dose dependent manner. Amiloride also blocks the 5-HT facilitation in the absence of concanavalin A. 5 to 6 wedges were used for each treatment. *, $p < 0.05$, 5-HT with and without amiloride; **, $p < 0.01$, concanavalin A plus 5-HT with and without amiloride; ***, $p < 0.001$, concanavalin A plus 5-HT vs 5-HT. φ, $p < 0.001$, NMDA plus amiloride vs. NMDA control.

phenylephrine (10 μ M) and carbachol (10 μ M) produced homologous desensitization as evidenced by a decreased response on second exposure 40 min following first exposure ($190 \pm 6\%$ vs $161 \pm 5\%$, $n=5$, and $214 \pm 38\%$ vs $176 \pm 30\%$, $n=6$ with phenylephrine and carbachol respectively) but the reduction was not statistically significant. When 5-HT 30 μ M was co-applied with NMDA, followed by phenylephrine 20 min later, no change in the phenylephrine facilitation was observed (Fig. 17). However, when 100 μ M 5-HT was applied first, the subsequent response to phenylephrine was reduced. Allowing 40 min to lapse between exposure to 5-HT and phenylephrine, no desensitization was evident. Similar results were obtained when 10 μ M carbachol was substituted for phenylephrine (Fig. 18).

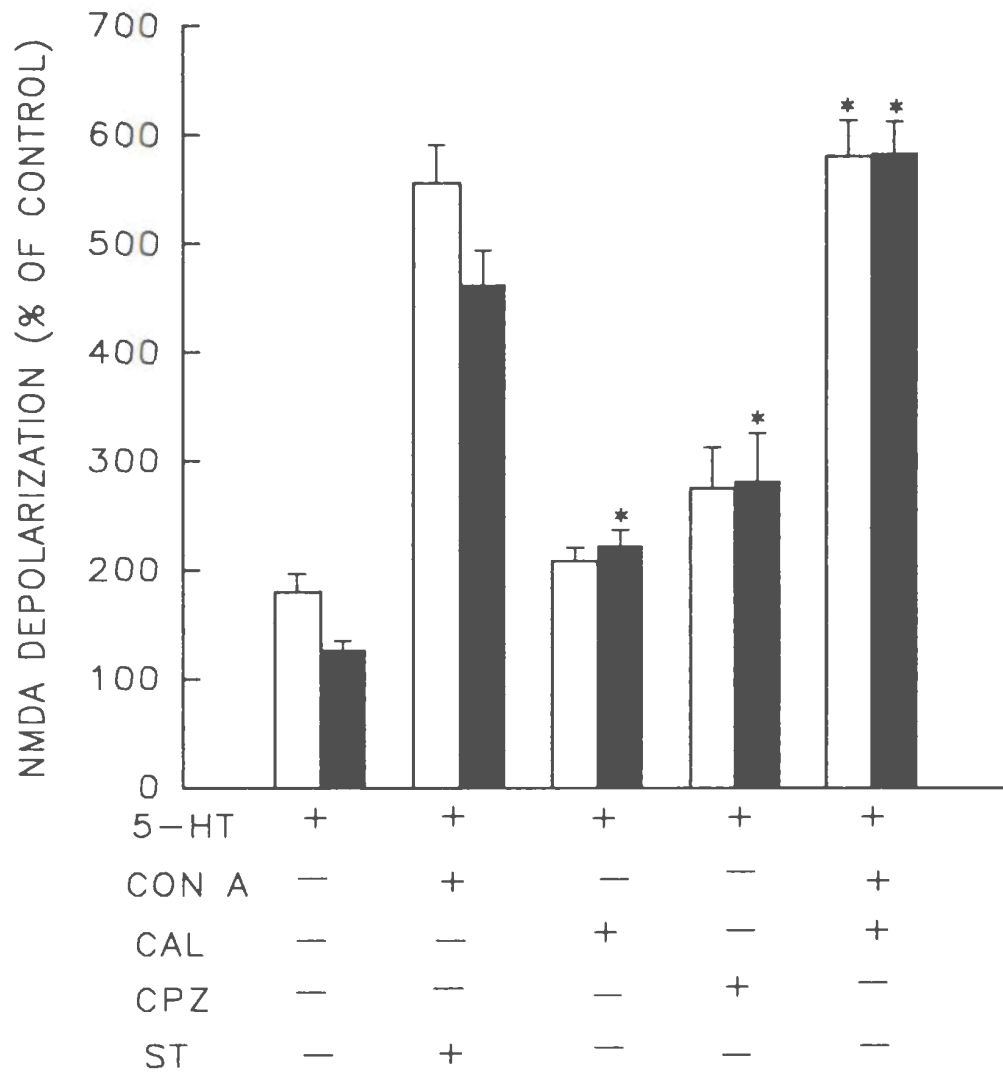


Figure 16. Calmodulin antagonists calmidazolium (CAL; 1 μ M) and chlorpromazine (CPZ; 1 μ M), but not concanavalin A plus staurosporine, prevent long term desensitization of 5-HT facilitation. Open bars indicate first exposure, filled bars second exposure. 6 to 8 wedges were used for each condition. *, $p < 0.05$, 5-HT plus treatment vs. 5-HT.

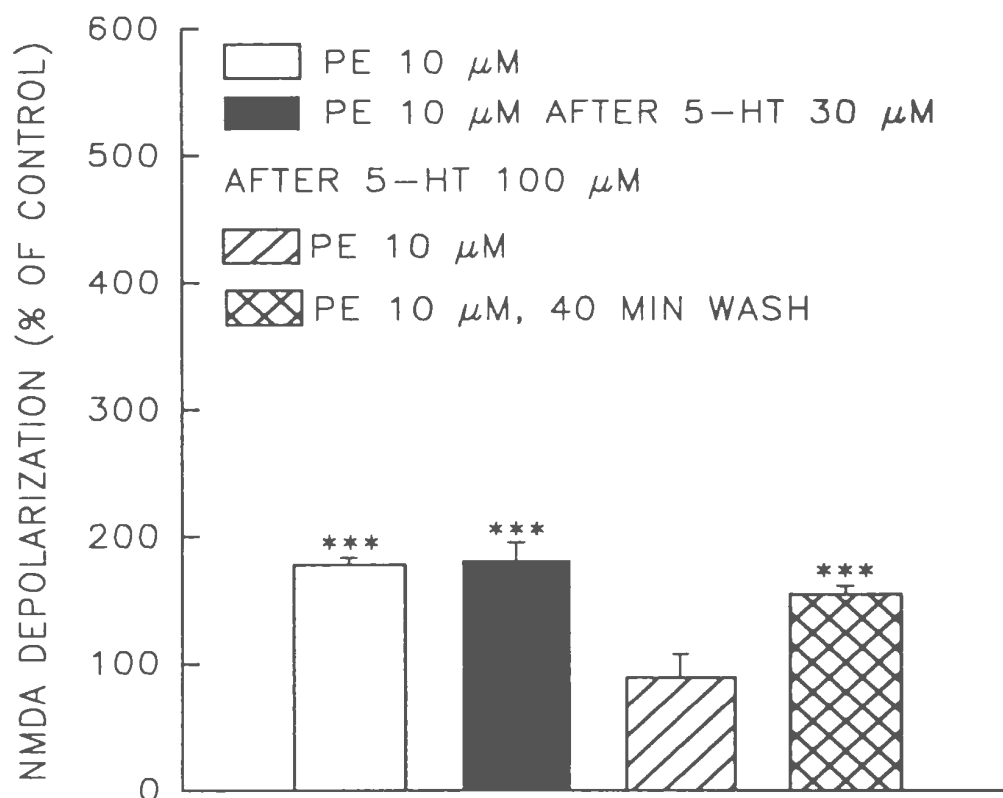


Figure 17. 5-HT induces heterologous desensitization of α_1 -adrenoceptor mediated facilitation. Desensitization of phenylephrine (PE) facilitation of the NMDA depolarization following prior exposure to 5-HT and NMDA is dependent on the concentration of 5-HT and the time between exposure to 5-HT and phenylephrine ***, $p < 0.001$, phenylephrine plus NMDA vs. NMDA control.

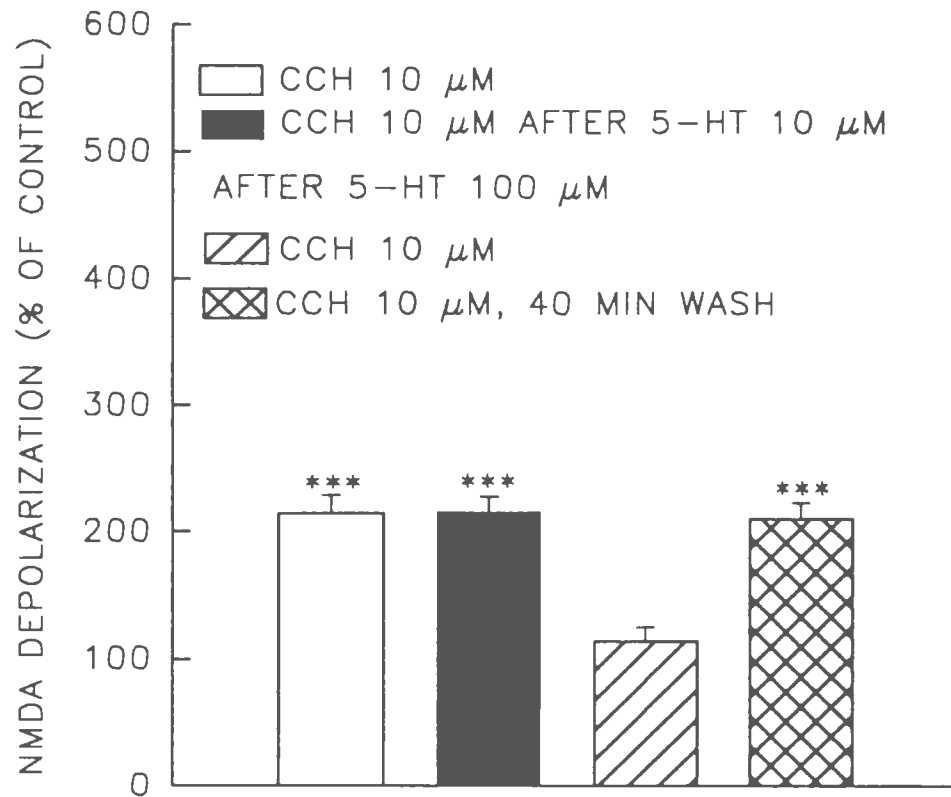


Figure 18. 5-HT induces heterologous desensitization of muscarinic cholinceptor-mediated facilitation. Desensitization of carbachol (CCH) facilitation of the NMDA depolarization following prior exposure to 5-HT and NMDA is dependent on concentration of 5-HT and time between exposure to 5-HT and carbachol ***, $p < 0.001$, carbachol plus NMDA vs. NMDA control.

3.4 Discussion

The present results reveal 5-HT receptor mediated enhancement of the NMDA depolarization undergoes acute desensitization. This is apparent on first exposure to the agonist when compared to wedges treated with staurosporine or concanavalin A. The 5-HT facilitation also undergoes long term desensitization, which becomes apparent on second exposure to agonist. Finally, 5-HT receptor activation results in heterologous desensitization of α_1 -adrenoceptors and muscarinic cholinceptors. In contrast, the depolarization induced by NMDA alone showed neither desensitization nor modification by protein kinase C activators and inhibitors, concanavalin A, or calmodulin antagonists. This suggests that desensitization reflects changes at 5-HT receptors and/or their associated transduction pathways. Furthermore, since the desensitization observed with DOI is similar in many respects to that found with 5-HT, the desensitization likely results in large part from an alteration in the 5-HT_{2A} receptor and its second messenger pathway. This suggestion is in keeping with observations on the neuronal depolarization mediated by 5-HT_{2A} receptors on cortical neurons. This depolarization also undergoes desensitization on repeated exposure to an agonist (Araneda and Andrade, 1991).

3.4.1 Apparent acute desensitization resulting from protein kinase C activation

Two types of acute desensitization were operative, one sensitive to protein kinase C, the other sensitive to concanavalin A. Thus, activating protein kinase C with oleoacetyl glycerol or phorbol diacetate totally suppressed the 5-HT facilitation. This action appears to be specific in that pretreatment with the protein kinase C inhibitor, staurosporine, restores the facilitation. On the other hand, inhibiting protein kinase C with staurosporine potentiates the 5-HT and DOI enhancement. Consistent with this finding, another protein kinase C inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), potentiates acetylcholine facilitation of the NMDA depolarization in hippocampal neurons (Markram and Segal, 1992). Potentiation, following protein kinase C inhibition, is congruous with 5-HT_{2A} mediated responses at facial motoneurons, smooth muscle, and platelets (Aghajanian, 1990; Roth et al., 1986; Kagaya *et al.*, 1990). Although staurosporine is not selective with respect to protein kinase C inhibition (IC₅₀ = 2.7 nM for protein kinase C vs. 6.7 nM for protein kinase A; Ruegg and Burgess, 1989), when taken together with the phorbol diacetate and oleoacetyl glycerol data, the most parsimonious explanation is that activation of protein kinase C contributes to desensitization of the 5-HT_{2A} receptor mediated enhancement.

In facial motoneurons, activation of protein kinase C reduces the agonist response, but not agonist independent activation of the G-protein coupled to the 5-

HT receptor. Thus, the receptor appears to be phosphorylated by protein kinase C (Aghajanian, 1990). Consistent with this view, the presumptive intracellular domains of the 5-HT_{2A} receptor have three regions with homology to protein kinase C consensus sequences for phosphorylation (Roth *et al.*, 1990). However, protein kinase C also phosphorylates the inositol 1,4,5-triphosphate (IP₃) receptor (Worley *et al.*, 1987). Activation of the IP₃ receptor releases intracellular Ca⁺⁺ (Berridge, 1987). It may be that a rise in intracellular Ca⁺⁺ is essential for 5-HT facilitation of the NMDA depolarization at cortical neurons as has been suggested for muscarinic cholinergic facilitation of NMDA induced depolarization at hippocampal neurons (c.f. Markram and Segal, 1991, 1992 and also see Chapter 5). Accordingly, phosphorylation of the IP₃ receptor might well contribute to blockade of the 5-HT facilitation.

Whether the 5-HT_{2A} receptor and/or the IP₃ receptor are phosphorylated, the present results are consistent with the notion that agonist induced protein kinase C activation results in negative feedback to reduce 5-HT_{2A} receptor mediated responses (Aghajanian, 1990; Roth *et al.*, 1986). However, additional cellular processes, unrelated to 5-HT_{2A} receptor activation, may also regulate protein kinase C associated with 5-HT facilitation. Since the present results with staurosporine do not distinguish between these possibilities, this issue should be left open and the processes referred to as "apparent" acute desensitization.

3.4.2 Acute desensitization resulting from receptor sequestration

Staurosporine only partially reversed desensitization at higher 5-HT concentrations. Indeed, in the presence of staurosporine there remains a concentration-dependent decline in the facilitation at elevated 5-HT concentrations; also, the inflection point for the decline is shifted to the left. This suggests the potentiated response promotes development of additional desensitization. In contrast to staurosporine, concanavalin A potentiated the 5-HT enhancement at 100 μ M, but not at lower concentrations. Concanavalin A prevents desensitization of 5-HT₄ (Ansanay *et al.*, 1992), kainate, glutamate receptor (see Lin and Levitan, 1991) and β -adrenoceptor mediated responses (Toews *et al.* 1984). In the case of β -adrenoceptors, concanavalin A reduces desensitization by preventing receptor sequestration or internalization which normally takes place at elevated agonist concentrations (Waldo *et al.*, 1983). Concanavalin A could well potentiate the 5-HT_{2A} response by preventing sequestration.

However, an alternative explanation is possible. Concanavalin A elevates intracellular pH (Mironov and Lux, 1991), which, by increasing the affinity of the IP₃ receptor for IP₃, could increase release of intracellular Ca⁺⁺ (Joseph *et al.*, 1989). Elevated Ca⁺⁺ would potentiate 5-HT enhancement of the NMDA depolarization (see Chapter 5). Moreover, increased intracellular Ca⁺⁺ mimics muscarinic cholinergic mediated enhancement of the NMDA depolarization in

hippocampal neurons (Markram and Segal, 1991, 1992). Amiloride, an inhibitor of the Na^+/H^+ -antiporter (Schellenberg *et al.*, 1983; Siffert and Akkerman, 1987) occludes the pH effect of concanavalin A (Mironov, 1992) and indeed a dose-dependent decline in the concanavalin A effect on the 5-HT response was observed during application of amiloride. However, the response to 5-HT alone was also suppressed. These results suggest that the pH lowering effect of amiloride may well explain loss of the 5-HT enhancement, but not the potentiation due to concanavalin A. If the effect of concanavalin A is simply on intracellular pH, it should potentiate the 5-HT facilitation at all 5-HT concentrations. That concanavalin A only potentiates the response to 5-HT at 100 μM implies it is selective for desensitization occurring at high agonist concentrations. This finding is compatible with the present result that decline of the 5-HT enhancement shifts to the left when the facilitation is potentiated by staurosporine. This would be expected for a receptor that undergoes response-dependent sequestration and therefore it is concluded that cortical 5-HT_{2A} receptors undergo internalization. Clearly, appropriate binding studies are necessary to confirm this conclusion.

3.4.3 Long term desensitization

Exposure to 5-HT and DOI resulted in long term desensitization as evidenced by the smaller response on second exposure to the agonist. Long term

desensitization induced by DOI is abolished by staurosporine, whereas it is eliminated by calmodulin antagonists, but not staurosporine in the case of 5-HT. This might reflect different consequences of 5-HT_{2A} receptor activation by full and partial agonists and/or that a 5-HT receptor other than the 5-HT_{2A} subtype contributes to long term desensitization, e.g. the 5-HT_{1A} receptor subtype. Since the IP₃ receptor is phosphorylated by Ca²⁺ calmodulin-dependent protein kinase II (Worley *et al.*, 1987), this is clearly a site worthy of further investigation in this regard.

3.4.4 Heterologous desensitization

Muscarinic cholinceptors, α_1 -adrenoceptors and 5-HT_{2A} receptors all couple to phospholipase C and the resulting second messenger cascade (Chuang, 1989), which may account for their common action in facilitating NMDA depolarization (Chapter 2) and producing homologous desensitization. The present finding that 5-HT produces heterologous desensitization of the phenylephrine and carbachol mediated facilitation suggests 5-HT_{2A}, α_1 -adrenoceptors, and muscarinic cholinceptors are located on the same cortical neurons. Given common second messengers and location on common neurons, the question arises as to how selectivity between activated receptors is maintained. In this study, heterologous desensitization differed from homologous desensitization in both duration of action

and concentration of 5-HT at which it was elicited. These results suggest 5-HT_{2A} receptors, α_1 -adrenoceptors and muscarinic cholinergic receptors are functionally, if not physically, compartmentalized. Whether these receptors functionally couple to the same or different NMDA receptors and the mechanism underlying heterologous desensitization remains to be determined.

The mechanism responsible for heterologous desensitization could be important for it may shed light on another question; i.e. does the 5-HT_{2A} receptor mediated facilitation of the NMDA depolarization depend on activating a PLC-PI dependent response? This issue is dealt with in the following chapter.

CHAPTER 4

MYO-INOSITOL REDUCES 5-HT_{2A} RECEPTOR INDUCED HOMOLOGOUS AND HETEROLOGOUS DESENSITIZATION

4.1 Introduction

In the preceding experiments (Chapter 3), the 5-HT, but not the NMDA component of the facilitation was shown to undergo homologous desensitization. In addition, prior exposure to elevated concentrations of 5-HT resulted in heterologous desensitization of α_1 -adrenoceptor and muscarinic cholinceptor induced facilitation of the NMDA response. Although multiple mechanisms contribute to homologous desensitization of 5-HT_{2A} receptors, the mechanism underlying 5-HT induced heterologous desensitization was not identified.

In the hippocampus, carbachol, likely acting via a second messenger following stimulation of phosphoinositide hydrolysis, induces delayed loss of firing (Pontzer and Crews, 1990; Pontzer *et al.*, 1992). Exogenous myo-inositol enhances this response and reduces its variability by ensuring substrate availability (Pontzer *et al.*, 1992). An adequate source of inositol is crucial to the synthesis of phosphoinositides and maintenance of signalling (Chuang, 1989). Since activation of cortical 5-HT_{2A}, α_1 -adrenoceptors and muscarinic cholinceptors also stimulates

PLC-PI (Chuang, 1989; Fisher *et al.*, 1992), exhaustion of substrate could explain 5-HT induced heterologous desensitization. Therefore, the effect of exogenous myo-inositol on the 5-HT induced facilitation and heterologous desensitization was examined.

4.2 Methods

The methods for wedge preparation, recording, drug application and data analysis were identical to those described in section 2.2 and 3.2. Myo-inositol (obtained from Sigma) was dissolved in water, diluted in ACSF and perfused throughout.

4.3 Results

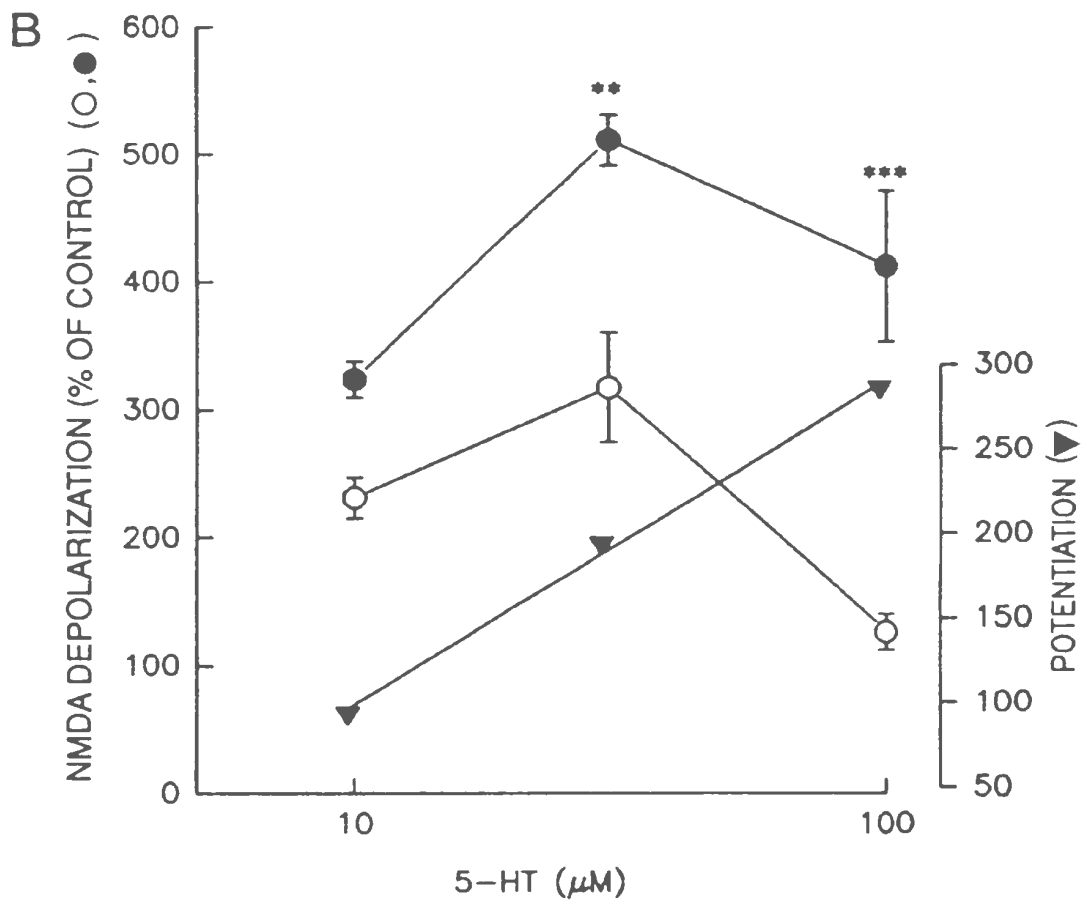
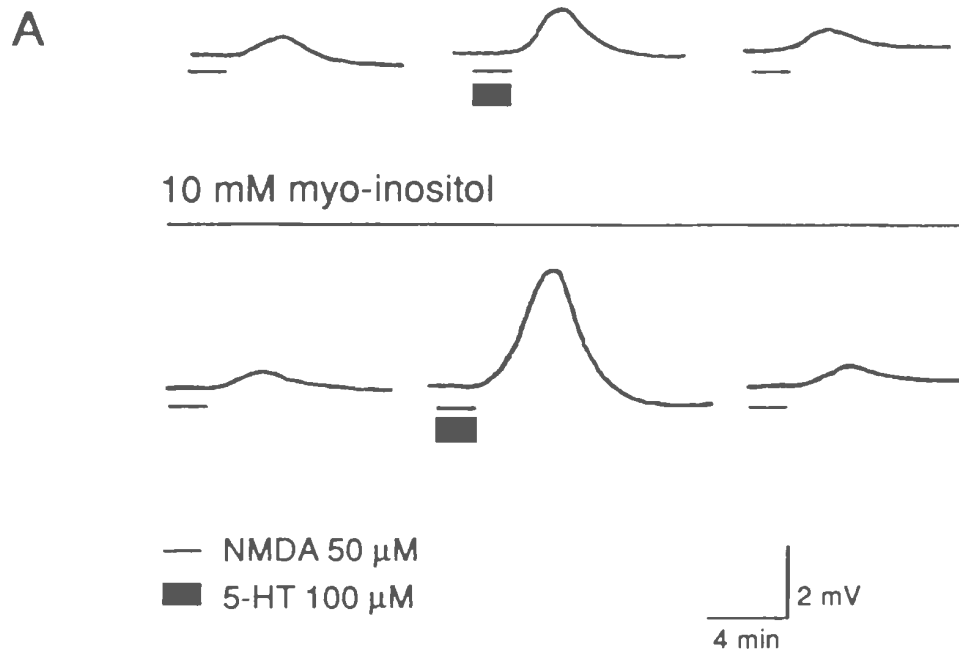
4.3.1 Potentiation of 5-HT facilitation by myo-inositol

In keeping with previous findings (Chapters 2 and 3), co-application of 5-HT (10 or 30 μ M) with NMDA significantly and reversibly enhanced the NMDA depolarization of rat cortical neurons, whereas little or no facilitation was observed when 100 μ M 5-HT was employed (Fig. 19). Perfusing 10 mM myo-inositol did not alter the NMDA depolarization ($89 \pm 5\%$ of control), whereas it potentiated the 5-HT facilitation (Fig. 19). The magnitude of the potentiation by myo-inositol increased linearly ($r=0.9986$, $r^2=0.9972$) as a function of 5-HT concentration (Fig. 19B). The slope of the resulting linear regression differed significantly from zero ($F=350$, $p=0.034$). Holding the concentration of 5-HT constant at 30 μ M, the potentiation varied with myo-inositol concentration (Fig. 20).

4.3.2 Myo-inositol and heterologous desensitization

Like 5-HT, co-application of carbachol or phenylephrine with NMDA resulted in a significant facilitation of the NMDA depolarization. This facilitation was absent when 100 μ M 5-HT was co-applied with NMDA 20 min earlier (Fig. 21). Perfusing 10 mM myo-inositol produced a small potentiation of the carbachol and phenylephrine induced facilitation and eliminated 5-HT induced heterologous desensitization (Fig. 21).

Figure 19. Myo-inositol potentiates 5-HT facilitation of NMDA depolarization. **A:** co-application of 5-HT (100 μ M) with NMDA (50 μ M) marginally increases amplitude of the NMDA depolarization (upper panel). Perfusing 10 mM myo-inositol (lower panel) potentiated the facilitation. Recovery (right; upper and lower panel) 20 min later. **B:** concentration-response relationship for 5-HT facilitation (left ordinate) in the absence (open circles) and presence of 10 mM myo-inositol (filled circles). The difference between the two curves (potentiation) is plotted below (closed triangles; right ordinate). Data from 4 to 7 wedges were averaged for each point. **, $p < 0.01$ and ***, $p < 0.001$, 5-HT vs. myo-inositol plus 5-HT.



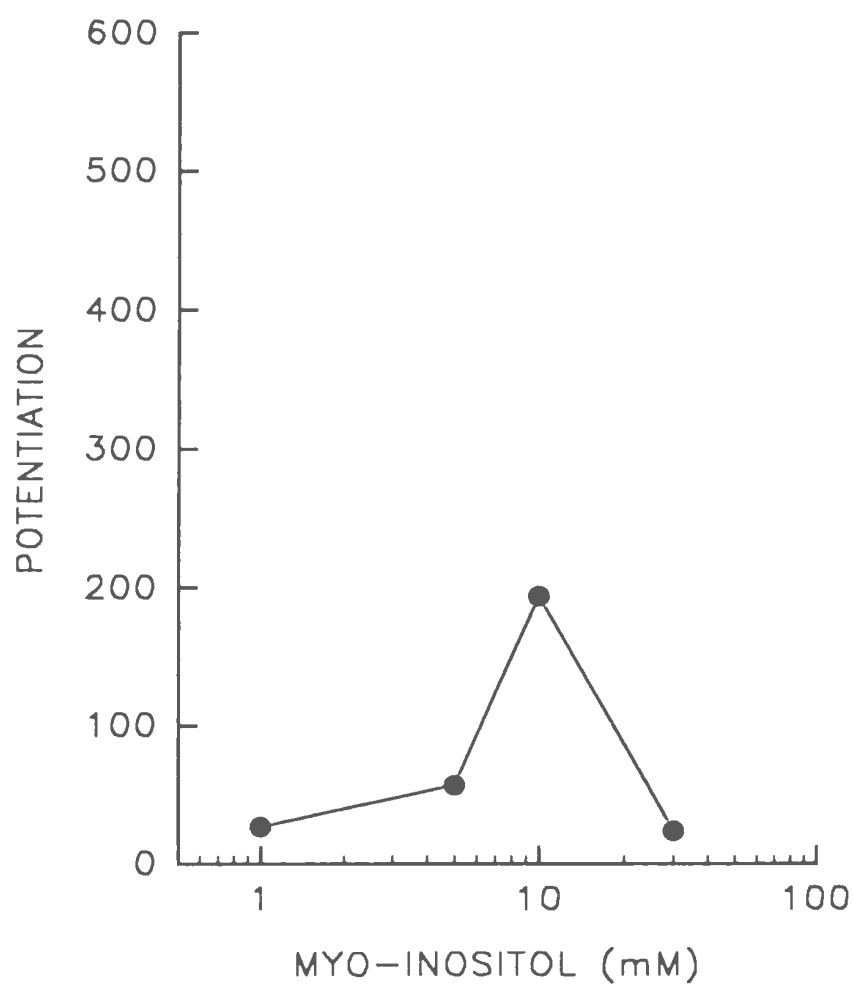
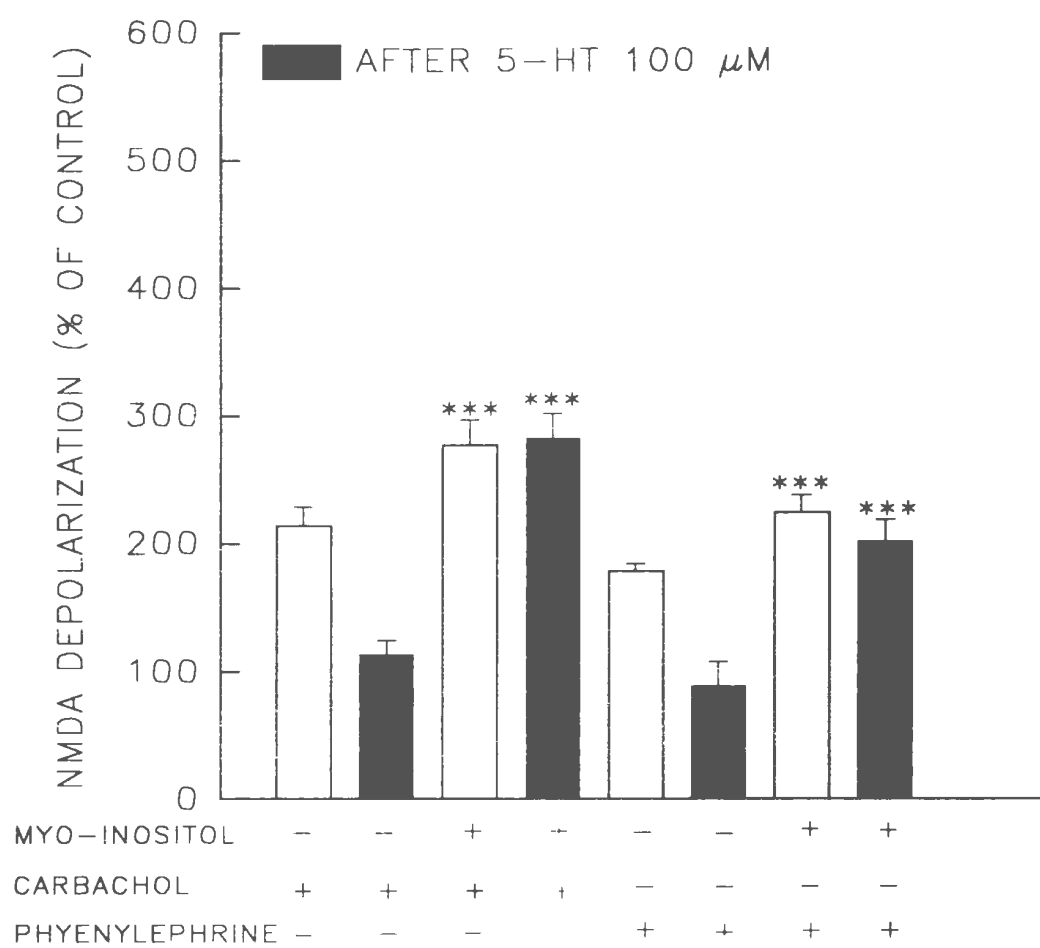


Figure 20. Myo-inositol concentration-response relationship. 5-HT concentration was constant at 30 μ M. Potentiation is the difference in response with and without myo-inositol. Each point is averaged from 4 to 7 wedges.

Figure 21. Myo-inositol eliminates 5-HT induced heterologous desensitization. Carbachol (10 μ M) and phenylephrine (10 μ M) facilitate the NMDA depolarization. Exposure to 100 μ M 5-HT 20 min before co-administration of carbachol or phenylephrine with NMDA significantly (n=5, *, p < 0.02) reduces the facilitation by the latter (heterologous desensitization). Perfusion of 10 mM myo-inositol prevents loss of response. 5 to 6 wedges per treatment. Open bars are control response obtained without prior exposure to 5-HT. ***, p < 0.001, myo-inositol vs control (without myo-inositol).



4.4 Discussion

4.4.1 Myo-inositol and 5-HT facilitation

Incubated brain slices exhibit lower inositol levels than fresh brain tissue unless maintained in medium containing 10 mM inositol (Sherman *et al.*, 1986). Moreover, perfusing myo-inositol results in greater inositol phosphate formation following carbachol stimulated phosphoinositide hydrolysis (Pontzer *et al.*, 1992). The 5-HT facilitation is mediated via 5-HT_{2A} receptors (Chapter 2) and activation of cortical 5-HT_{2A} receptors leads to PLC-PI response (Chuang, 1989; Fisher *et al.*, 1992). If the facilitation depends on 5-HT_{2A} receptors activating the PLC-PI response, then perfusion with myo-inositol could potentiate the 5-HT facilitation simply by increasing phosphoinositide substrate availability. This suggestion is in keeping with the findings that: 1) magnitude of the potentiation increases linearly with 5-HT concentration and; 2) potentiation varies with myo-inositol concentration.

Cortical 5-HT_{2A} receptors might undergo receptor internalization or sequestration which is a concentration dependent phenomenon (Chapter 3). Mechanistically, the large potentiation induced by myo-inositol at 100 μ M 5-HT is difficult to reconcile with 5-HT_{2A} receptors undergoing sequestration. Moreover, linearity of the potentiation is inconsistent with myo-inositol acting via multiple

mechanisms dependent on 5-HT concentration. Instead, it is likely that substrate availability and/or a product of phosphoinositide hydrolysis influences 5-HT_{2A} receptor sequestration. Thus, substrate exhaustion, favoured by the absence of exogenous myo-inositol, would lead to receptor sequestration. Clearly, appropriate binding studies are necessary to resolve this point.

4.4.2 Myo-inositol prevents heterologous desensitization

The observation that myo-inositol also potentiates the carbachol and phenylephrine induced facilitation implies that activation of 5-HT_{2A}, α_1 -adrenoceptors, and muscarinic cholinergic receptors might result in facilitation through activation of identical second messenger pathways. Moreover, the observation that 5-HT induces heterologous desensitization suggests that cortical projection neurons (pyramidal cells) share overlapping populations of these receptors (Chapter 3). Thus, depletion of phosphoinositide substrate by 5-HT could well account for the observed heterologous desensitization. Consistent with this interpretation are the findings that: 1) myo-inositol potentiation increases with 5-HT concentration and; 2) only at the highest 5-HT concentration employed (100 μ M), i.e. where the greatest depletion of substrate is expected, is heterologous desensitization present (Chapter 3).

In conclusion, perfusing cortical wedges with myo-inositol reduces homologous and heterologous desensitization. More important, these data further implicate involvement of PLC-PI response and the resulting second messenger cascade in the novel interaction between 5-HT_{2A} and NMDA receptors. The exclusion of protein kinase C involvement in the facilitation suggests the other pathway, i.e., IP₃-Ca⁺⁺ may underlie the 5-HT facilitation.

CHAPTER 5

5-HT_{2A} RECEPTOR MEDIATED FACILITATION OF THE NMDA

DEPOLARIZATION: THE ROLE OF Ca⁺⁺

5.1 Introduction

5-HT acting at 5-HT_{2A} receptors facilitates the NMDA depolarization of cortical projection (pyramidal) neurons by a direct action on those neurons (see Chapter 2). Although activation of 5-HT_{2A} receptors produces a small decrease in the resting K⁺ conductance and reduces the slow afterhyperpolarization (Davies *et al.*, 1987; Araneda and Andrade, 1991), the facilitation is unrelated to these effects as it is observed in the presence of K⁺ channel blockers, under voltage clamp conditions, and is not mimicked by agents which alone block the slow afterhyperpolarization (Reynolds *et al.*, 1988; see Chapter 3). The 5-HT_{2A} receptor is a member of the G-protein coupled receptor superfamily and in common with a number of other such receptors is positively coupled to phospholipase C (see section 1.2.6). In cortical neurons, facilitation of the NMDA depolarization by 5-HT likely results as a consequence of activating phospholipase C since myo-inositol: 1) potentiates the facilitation in a concentration dependent manner; 2) reduces homologous desensitization of the 5-HT_{2A} response and; 3) eliminates

heterologous desensitization of the carbachol and phenylephrine induced facilitation (see Chapter 4).

Stimulation of phospholipase C leads to the production of diacylglycerol and inositol 1, 4, 5 triphosphate (IP_3) (Berridge, 1993). Diacylglycerol in turn activates protein kinase C; in hippocampal neurons and *Xenopus* oocytes, stimulation of protein kinase C or introduction of the catalytic subunit of protein kinase C facilitates the NMDA response (Aniksztejn *et al.*, 1991, 1992; Kelso *et al.*, 1992). On the other hand, at cortical neurons, activation or inhibition of protein kinase C does not alter the depolarization induced by NMDA (Chapter 3).

The IP_3 produced by phospholipase C acts at IP_3 receptors on smooth endoplasmic reticulum to release Ca^{++} stored at this site (Taylor and Richardson, 1991; Berridge, 1993; Pozzan *et al.*, 1994). This rise in Ca^{++} mediated by IP_3 is thought to be essential for facilitation of the NMDA depolarization mediated by muscarinic cholinergic receptors (Markram and Segal, 1992). If a similar mechanism accounts for the 5-HT_{2A} facilitation at cortical neurons, then agents which raise the concentration of intracellular Ca^{++} without increasing IP_3 should also facilitate the NMDA depolarization. In order to test this hypothesis, a series of structurally unrelated agents, which share in common an ability to elevate intracellular Ca^{++} , were employed. Thapsigargin, a naturally occurring sesquiterpene lactone (Thastrup *et al.*, 1990), cyclopiazonic acid, an indole tetramic acid metabolite of

Aspergillus and *penicillium* (Seidler *et al.*, 1989), and A23187 were chosen for study. The mechanism(s) by which these agents increase intracellular Ca^{++} does not involve the generation of IP_3 . Rather, thapsigargin and cyclopiazonic acid selectively inhibit the endoplasmic reticulum Ca^{++} -ATPase (Thastrup *et al.*, 1990; Verma *et al.*, 1990; Mason *et al.*, 1991) and A23187 induces the formation of divalent cationic ionophores (Pressman, 1976).

5.2 Methods

5.2.1 Wedge preparation and recording

Wedges from sensorimotor cortex were prepared for recording and analyzed as described in section 2.2.2 and 2.2.5.

5.2.2 ACSF composition and drug application

ACSF composition and drug application were as described in sections 2.2.3, 2.2.4, and 3.2.2 with the following additions: thapsigargin, cyclopiazonic acid and A23187 were applied for 3 min (1 min alone prior to NMDA application and 2 min with NMDA). Increasing the perfusion time to 3 min for thapsigargin, cyclopiazonic acid and A23187 was found to produce a large, consistent facilitation when compared to the 2 min perfusion time used for 5-HT. The longer exposure time was therefore employed in all experiments. Thapsigargin, cyclopiazonic acid, A23187, BAPTA-AM and staurosporine were dissolved in dimethylsulfoxide and diluted in ACSF. Chlorpromazine and BAPTA-AM were perfused for 40 min before addition of agonists. Tetrodotoxin (0.1-0.3 μ M) was perfused throughout to minimize indirect effects. An antagonist cocktail consisting of D,L-AP3, scopolamine, ritanserin and prazosin was perfused 40 min before addition of agonists.

5.2.3 Drugs and chemicals

2-amino-3-phosphonopropionic acid (D,L-AP3), Bis-(o-aminophenoxy)-ethane-N,N,N',N', tetraacetic acid aminoethoxy (BAPTA-AM) and thapsigargin were from Calbiochem. Calcium ionophore A23187, cyclopiazonic acid, kainic acid, myo-inositol and nifedipine were obtained from Sigma. Drug concentrations were calculated as the salt. Stock solutions were kept frozen until use.

5.3 Results

5.3.1 Effects of thapsigargin, cyclopiazonic acid and A23187 on the NMDA depolarization

Perfusing NMDA depolarized cortical neurons and in keeping with previous observations (Chapters 2 and 3), 5-HT facilitated the NMDA depolarization (Fig. 22). Perfusing 100 nM thapsigargin alone did not alter the recorded potential (data not shown), but when combined with NMDA, it reversibly facilitated the NMDA depolarization (Fig. 22).

Like 5-HT and thapsigargin, cyclopiazonic acid and A23187 also facilitated the NMDA depolarization without modifying the potential when perfused alone. The concentration-response curves for the thapsigargin, cyclopiazonic acid and A23187 induced facilitation are shown in Fig. 23. Except for differences in potency and a greater efficacy for A23187, the curves are remarkably similar. All three agents facilitated the NMDA depolarization in a concentration dependent manner up to a maximum, followed by loss of the facilitation at the highest concentration.

5.3.2 Calmodulin antagonism and the A23187 facilitation

Loss of the facilitation at elevated concentrations of A23187, thapsigargin

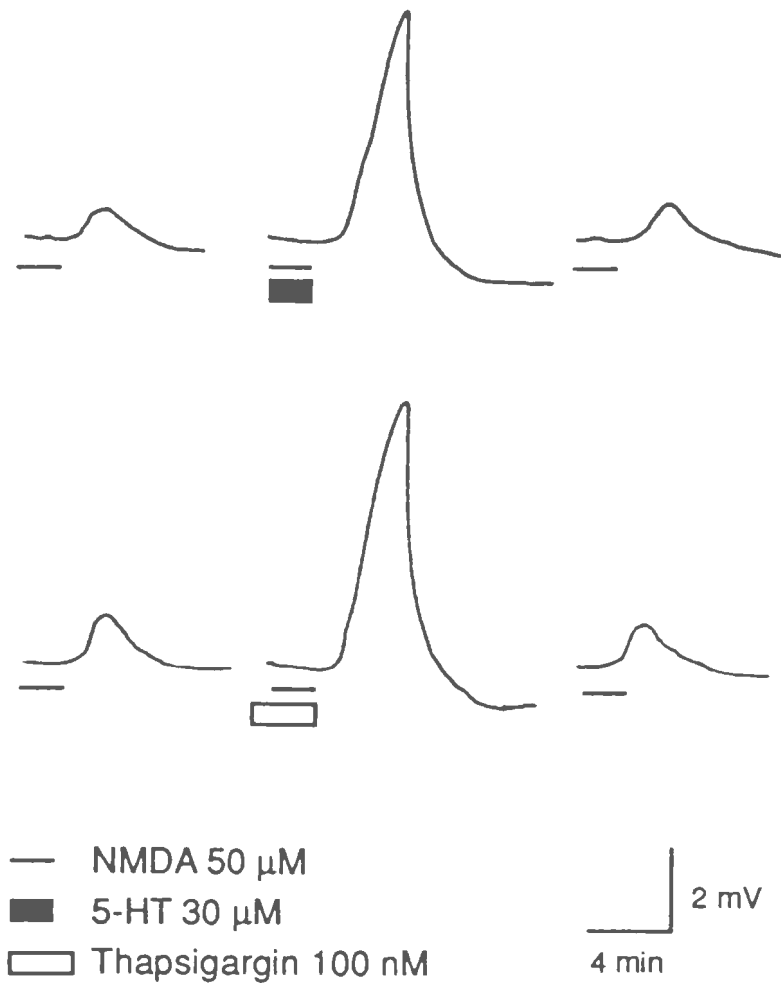


Figure 22. Facilitation of NMDA depolarization by 5-HT and thapsigargin. NMDA depolarizes cortical neurons with respect to corpus callosum (left). Administration of 5-HT (upper panel) and thapsigargin (lower panel) facilitates the NMDA response. Recovery 20 min later.

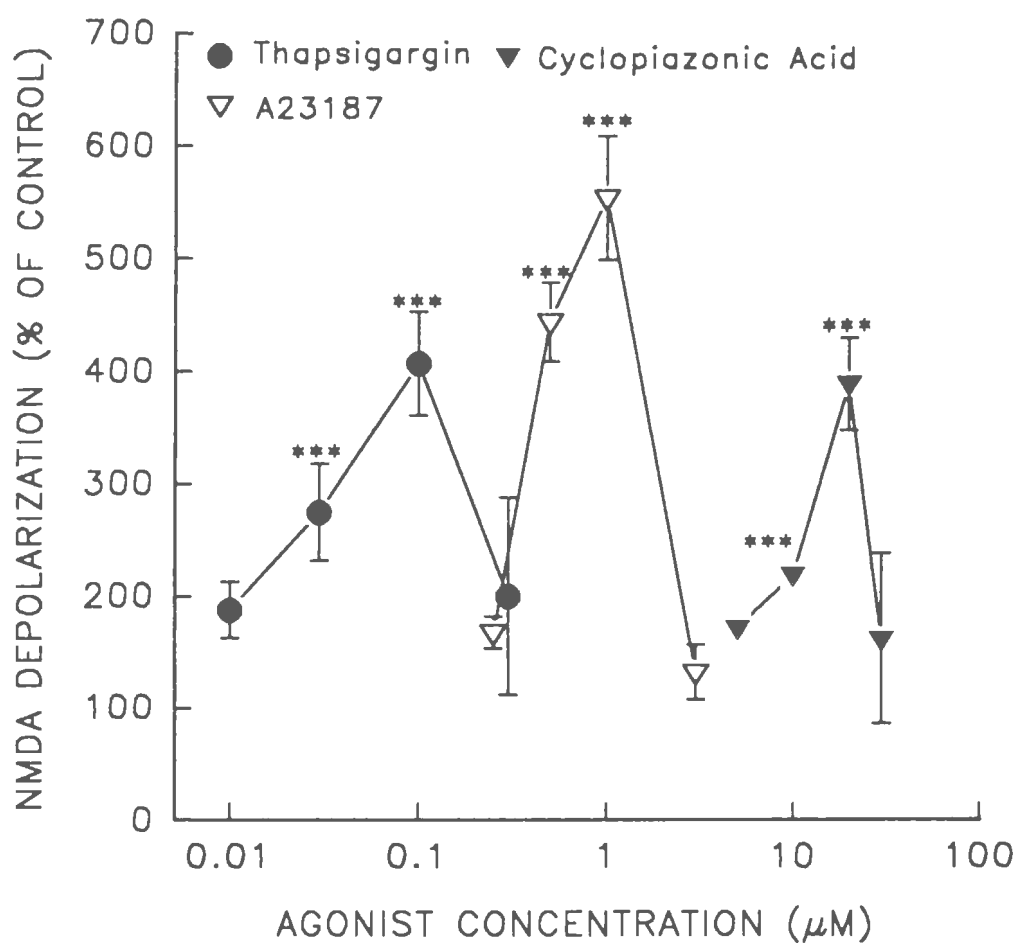


Figure 23. The concentration-response relationship for thapsigargin, cyclopiazonic acid and A23187. NMDA concentration was 50 μM . 5 to 8 wedges were tested at each concentration. **, $p < 0.01$; ***, $p < 0.001$; NMDA vs treatment.

and cyclopiazonic acid may represent some form of desensitization, perhaps triggered by the large increase in intracellular Ca^{++} concentration expected with these agents (Kennedy, 1989). Calmodulin is activated by a rise in intracellular Ca^{++} (Erondy and Kennedy, 1985) and one form of desensitization of the 5-HT_{2A} facilitation in cortical neurons is blocked by calmodulin antagonists (Chapter 3). Perfusing chlorpromazine $1\ \mu\text{M}$, a calmodulin antagonist, for 40 min before exposure to A23187 and NMDA partially reduced loss of the facilitation (Fig. 24). A similar effect was observed when $20\ \mu\text{M}$ cyclopiazonic acid and $0.3\ \mu\text{M}$ thapsigargin were substituted for A23187 (data not shown). Other drugs which alter the 5-HT_{2A} receptor desensitization including staurosporine and myo-inositol did not alter loss of the facilitation observed with A23187 (data not shown).

5.3.3 Facilitation is selective for the NMDA depolarization

Application of 5-HT facilitates the NMDA depolarization, but not that induced by quisqualate and kainate (see Chapter 2). When cyclopiazonic acid ($10\ \mu\text{M}$) was perfused with a submaximal concentration of quisqualate or kainate ($5\ \mu\text{M}$), no facilitation of the depolarization took place ($103 \pm 4\%$, $n=4$ and $92 \pm 4\%$, $n=3$ respectively, Fig. 25). Further trials revealed cyclopiazonic acid ($20\ \mu\text{M}$, $n=2$) also failed to facilitate the depolarization induced by quisqualate (data not shown).

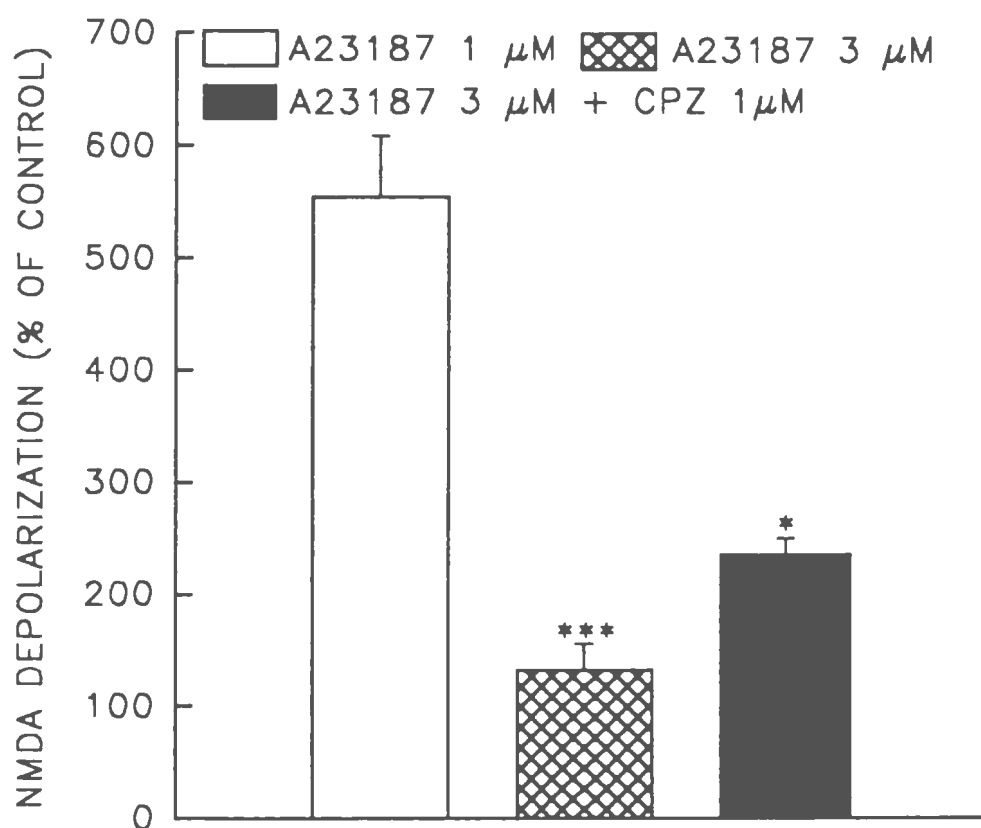


Figure 24. Calmodulin antagonist chlorpromazine reduces loss of A23187 facilitation. Chlorpromazine (CPZ) did not affect the NMDA depolarization. 4 to 5 wedges were used for each treatment. *, $p < 0.05$; treatment vs 3 μ M A23187.

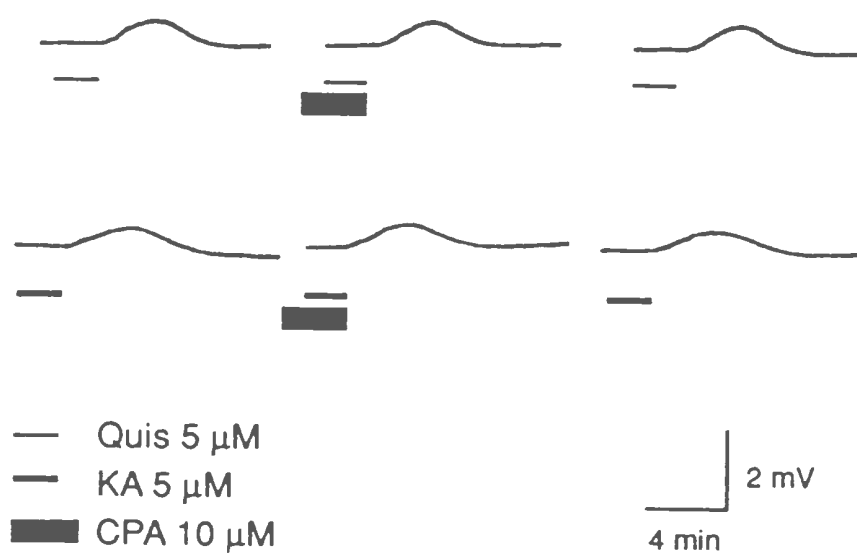


Figure 25. Depolarization of cortical neurons by quisqualate (Quis) and kainate (KA) is not enhanced by cyclopiazonic acid (CPA) administration.

5.3.4 Effects related to transmitter release

In cortical wedges, the amplitude of the NMDA depolarization is influenced by release of acetylcholine and norepinephrine (Chapter 2). Despite the presence of TTX, increasing the concentration of intracellular Ca^{++} in nerve-terminals and varicosities might increase the release of transmitters/modulators that facilitate the NMDA depolarization on pyramidal neurons, i.e. the facilitation could be indirect. To test this possibility, A23187, cyclopiazonic acid and thapsigargin were applied in the presence of a "Cocktail" consisting of prazosin (1 μM), scopolamine (10 nM), D,L-AP3 (50 μM) and ritanserin (10 nM) to antagonize α_1 -adrenoceptors, muscarinic cholinergic receptors, metabotropic glutamate receptors and 5-HT_{2A} receptors, respectively. Stimulation of all these receptors is known to facilitate the NMDA depolarization (see Chapters 2 and 7). Perfusing this antagonist cocktail reduced the amplitude of NMDA depolarization (Fig. 26). Moreover, the magnitude of the facilitation in the presence of thapsigargin, cyclopiazonic acid and A23187 was reduced when compared to the control facilitation (Fig. 26), although in all cases facilitation remained significant.

5.3.5 Ca^{++} dependence of the facilitation

Omitting Ca^{++} from the ACSF significantly reduces the 5-HT and norepinephrine induced phosphoinositide hydrolysis in cortical slices (Kendall and

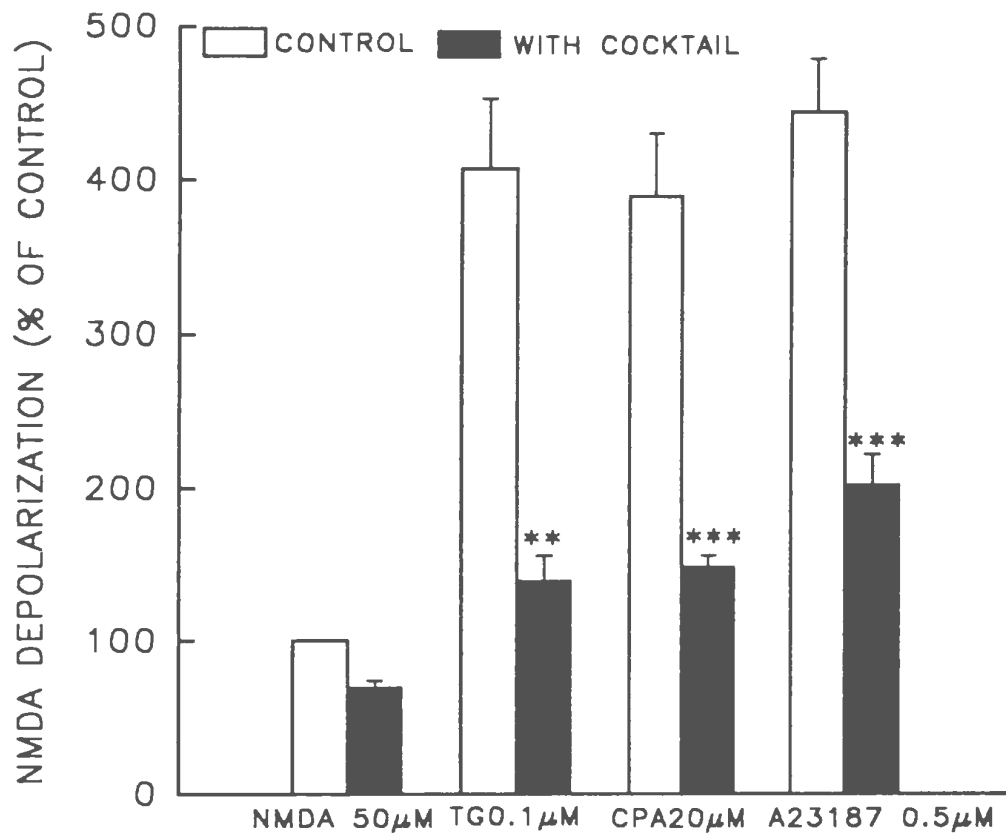


Figure 26. A cocktail consisting of TTX (0.3 μ M), scopolamine (10 nM), prazosin (1 μ M), ritanserin (10 nM) and D,L-AP3 (50 μ M) reduced, but did not abolish the facilitation by thapsigargin (TG), cyclopiazonic acid (CPA) and A23187. 5 to 6 wedges were used for each treatment. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; cocktail vs cocktail plus drug.

Nahorski, 1984). Similarly, omitting Ca^{++} from the ACSF eliminates the phenylephrine induced facilitation of the NMDA depolarization (see Chapter 7). As shown in Fig. 27, when 5-HT was applied with NMDA during perfusion with Ca^{++} free ACSF (no added Ca^{++}) throughout, the facilitation was eliminated. In 5 wedges so treated, the average response amplitude was $99 \pm 6\%$ of the NMDA control. Perfusing BAPTA-AM, a Ca^{++} chelator which is only effective in chelating intracellular Ca^{++} (Niesen *et al.*, 1991) leads to the same result, i.e. the 5-HT facilitation was eliminated ($110 \pm 7\%$ of control, $n=5$, Fig. 27). Perfusing BAPTA-AM also reduced the amplitude of the NMDA depolarization ($61 \pm 6\%$, $n=5$, $p < 0.05$). Perfusing thapsigargin in Ca^{++} free ACSF or in the presence of BAPTA-AM yielded results similar to those obtained with 5-HT, i.e. in both cases the facilitation was eliminated (Fig. 27).

5.3.6 Nifedipine blocks facilitation of the NMDA depolarization

Ca^{++} can enter neurons through a variety of voltage- and receptor- operated channels (Kullman *et al.*, 1992). The L-type Ca^{++} channel is present on cortical pyramidal neurons and is blocked by nifedipine (Sayer *et al.*, 1992). As illustrated in Fig. 28, nifedipine concentration-dependently reduced the 5-HT and the A23187 induced facilitation. Similar results were obtained when 30 nM thapsigargin and 10 μM cyclopiazonic acid were substituted for A23187 (data not shown).

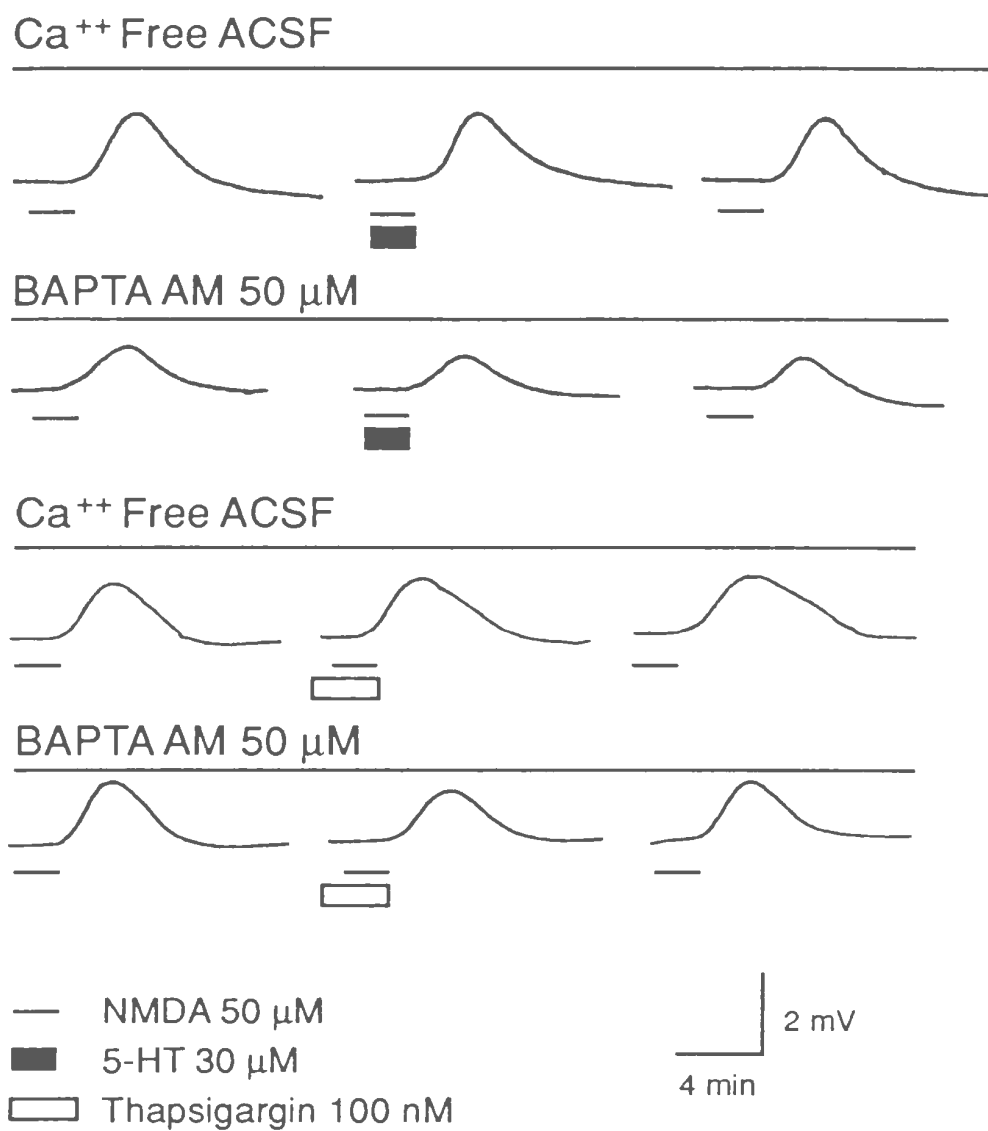
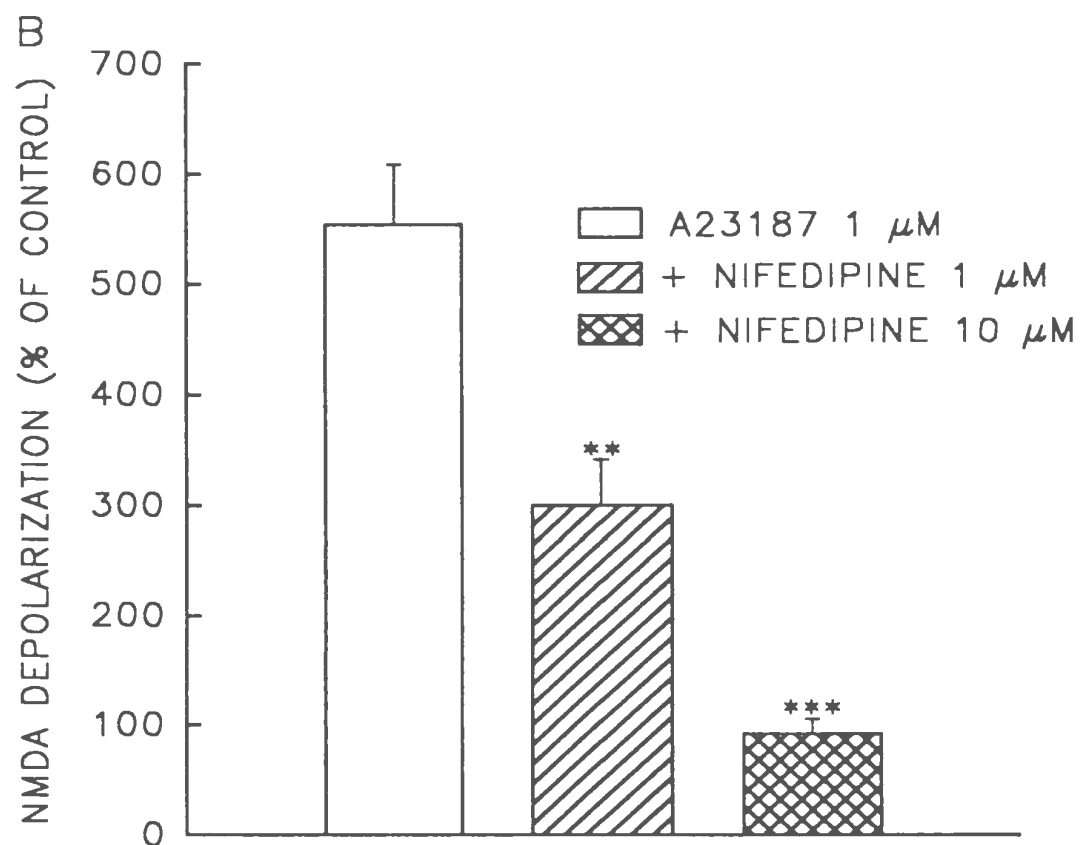
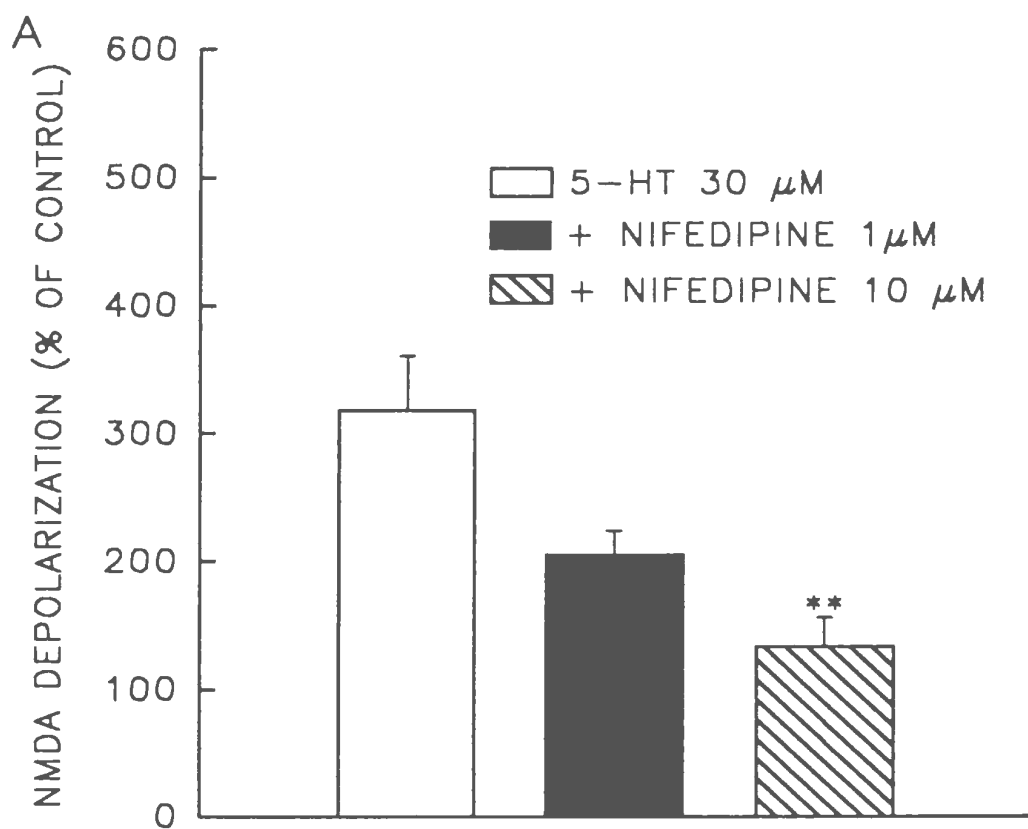


Figure 27. Perfusion of either Ca⁺⁺ free ACSF (no added Ca⁺⁺) or BAPTA-AM eliminates 5-HT and thapsigargin facilitation.

Figure 28. Nifedipine reduces the facilitation by 5-HT and A23187 in a concentration-dependent manner. 5 to 6 wedges were used at each concentration.

******, $p < 0.01$; *******, $p < 0.001$; nifedipine vs control.



5.3.7 Mg^{++} free medium does not eliminate the NMDA facilitation

The NMDA receptor ionophore exhibits a voltage-dependent conductance which is dependent on Mg^{++} . If the facilitation results from altering the Mg^{++} sensitivity (Chen and Huang, 1992), then removal of Mg^{++} should abolish the facilitation. When 5-HT was applied after perfusing the tissue for 1 hr with Mg^{++} free ACSF the facilitation was abolished ($100 \pm 1\%$ of control, $n=6$; Fig. 29). The phenylephrine facilitation exhibited a similar Mg^{++} sensitivity ($99 \pm 9\%$, $n=6$). In contrast, the A23187 induced facilitation persisted in Mg^{++} free ACSF ($318 \pm 43\%$ of control, $n=4$; Fig. 29B).

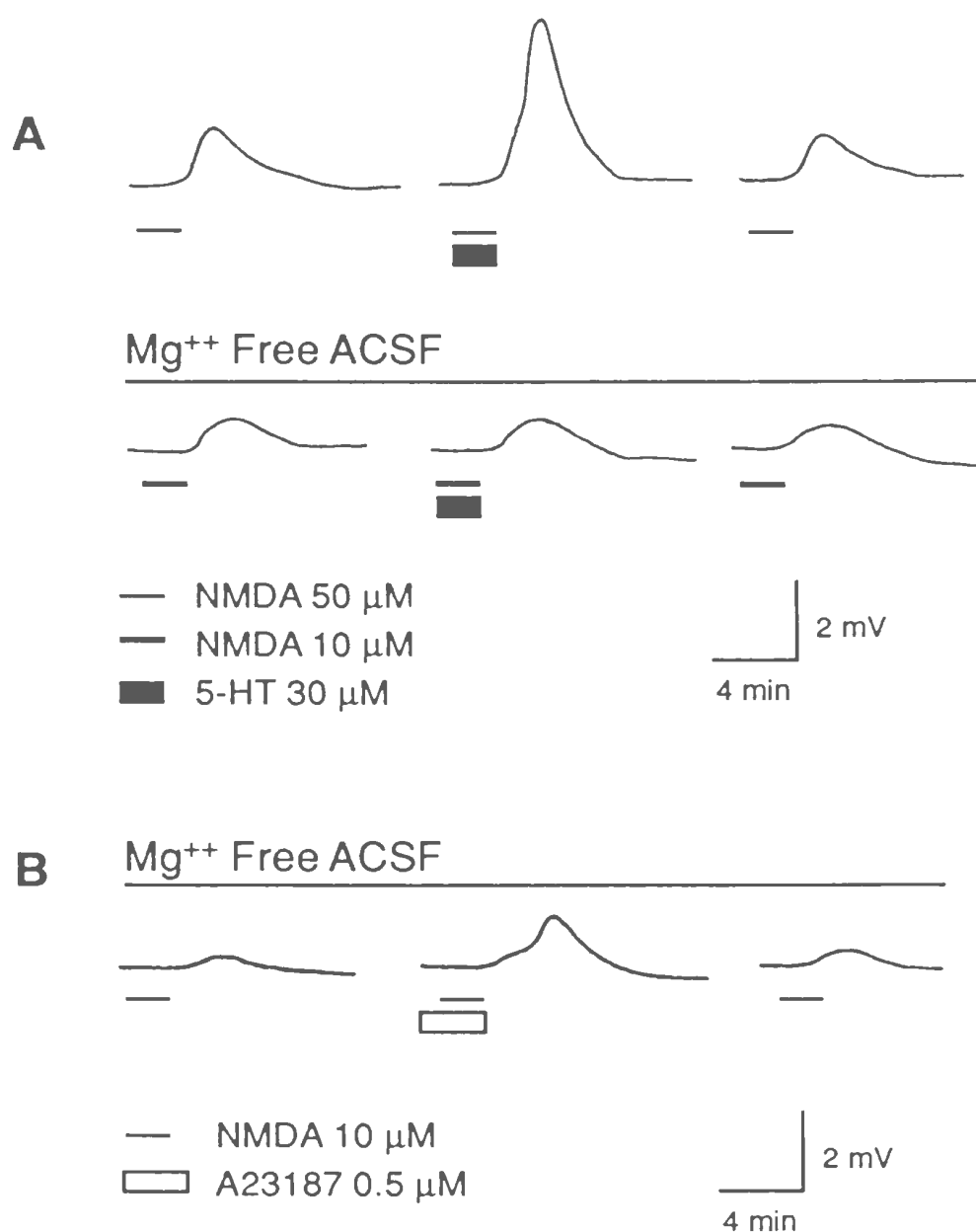


Figure 29. Effect of perfusing Mg⁺⁺ free ACSF (no added Mg⁺⁺) on the 5-HT and A23187 facilitation. **A.** 5-HT facilitates NMDA depolarization (upper panel), whereas this facilitation is absent in Mg⁺⁺ free medium (lower panel). **B.** A23187 facilitation persists in Mg⁺⁺ free medium.

5.4. Discussion

The major findings of this study are: 1) thapsigargin, cyclopiazonic acid, and A23187 induce facilitation of the NMDA depolarization, which, in part, is likely a direct effect and which mimics the 5-HT facilitation; 2) antagonism of calmodulin reduces loss of facilitation that occurs at elevated concentrations of agents used to raise intracellular Ca^{++} ; 3) both extracellular and intracellular Ca^{++} are critical for the facilitation observed in the present study; 4) the facilitation does not involve modification of the Mg^{++} dependent voltage sensitivity of the NMDA receptor ionophore. Therefore, the present study supports the hypothesis that 5-HT, acting at 5-HT_{2A} receptors (see section 5.1), elevates intracellular Ca^{++} which accounts for the NMDA facilitation. However, a major question remains, namely how does an elevation of intracellular Ca^{++} translate into facilitation of the NMDA response?

Thapsigargin and cyclopiazonic acid both raise the concentration of intracellular Ca^{++} by inhibiting an endoplasmic reticulum Ca^{++} -ATPase necessary to fill the IP_3 sensitive Ca^{++} pool (Thastrup *et al.*, 1990; Verma *et al.*, 1990; Mason *et al.*, 1991). This pool, located in the smooth endoplasmic reticulum, displays a constant leak of Ca^{++} so that inhibiting the pump results in loss of Ca^{++} from the storage site (Pozzan *et al.*, 1994). Thapsigargin and cyclopiazonic acid differ in their action from receptors coupled to phospholipase C in that the rise in Ca^{++} they

induce is not accompanied by a rise in inositol 1,4,5 triphosphate (Thastrup *et al.*, 1990; Jackson *et al.*, 1988). Except for a difference in potency, the facilitation induced by thapsigargin and cyclopiazonic acid is remarkably similar, consistent with a common mechanism of action. On the other hand, A23187 demonstrates greater efficacy with respect to facilitating the NMDA response than does thapsigargin or cyclopiazonic acid. This likely reflects the mechanism by which A23187 increases intracellular Ca^{++} . A23187 forms divalent selective cation ionophores which allow Ca^{++} to enter the cytosol from the extracellular space as well as from Ca^{++} containing intracellular organelles (Pressman, 1976; Pozzan *et al.*, 1994). A23187 may also raise the level of Ca^{++} indirectly by stimulating phosphoinositide hydrolysis (Fisher and Agranoff, 1981; Brammer *et al.*, 1988; Brammer and Weaver, 1989), although it exhibits a different pattern of inositol polyphosphate formation than do receptors coupled to phospholipase C (Baird and Nahorski, 1990).

5.4.1 Are the effects of thapsigargin, cyclopiazonic acid and A23187 direct?

Critical to understanding how thapsigargin, cyclopiazonic acid and A23187 facilitate the NMDA depolarization is knowing whether the facilitation induced by these agents results indirectly through transmitter/modulator release. In cortical wedges, scopolamine and prazosin significantly reduce the amplitude of the NMDA

depolarization, suggesting that tonic or more probably phasic release of acetylcholine and norepinephrine results in enhancement of the NMDA response (Chapter 2). Thus, thapsigargin, cyclopiazonic acid and A23187 might simply enhance the postsynaptic NMDA response by raising the concentration of Ca^{++} in presynaptic terminals and varicosities thereby increasing transmitter/modulator release (c.f. Markram and Segal, 1991). Indeed, the observation that a mixture of receptor antagonists dramatically reduces the facilitation strongly suggests that transmitter/modulator release contributes substantially to the facilitation observed with thapsigargin, cyclopiazonic acid, and A23187. Moreover, it could be argued that the significant facilitation remaining in the presence of the antagonists mixture might result from the action of yet another transmitter/modulator for which an antagonist was not present. Although such an interpretation cannot be excluded it appears improbable.

First, the concentration-response curves for thapsigargin, cyclopiazonic acid, and A23187 are quite similar, i.e., the facilitation rises to a peak after which it rapidly returns to the control value. If the facilitation resulted solely from transmitter/modulator release, it is hard to account for the shape of the concentration-response curve. However, an increasing contribution of an inhibitory transmitter, i.e. GABA, could account for this reduction and cannot be ruled out. Thus, if the mechanism was simply enhanced transmitter/modulator

release then the facilitation might be expected to show a plateau or perhaps a continuous increase rather than a return to baseline. Agonist induced facilitation up to 700% has been observed with 5-HT (see Chapter 3) demonstrating that larger responses than those observed with thapsigargin, cyclopiazonic acid and A23187 are possible.

Second, the 5-HT facilitation, but not that due to A23187, is eliminated when the tissue is perfused with Mg^{++} free ACSF. As suggested below, this may reflect the importance of Mg^{++} in G-protein coupling and not for the facilitation *per se*. If this is the case, then the facilitation induced by A23187 in Mg^{++} free ACSF is incompatible with release of a G-protein coupled transmitter/modulator being solely responsible for the facilitation. Indeed, the reduction in A23187 facilitation in Mg^{++} free ACSF that took place when compared to the facilitation in the presence of Mg^{++} may reflect loss of the G-protein receptor contribution.

Third, neither the carbachol nor the 1S,3R-ACPD facilitation are eliminated in Ca^{++} free ACSF (see Chapter 7). Moreover, nifedipine does not block the 1S,3R-ACPD facilitation (Chapter 7). However, the facilitation induced by thapsigargin and A23187 is absent in Ca^{++} free ACSF or in the presence of nifedipine. Thus, if the primary action of thapsigargin and A23187 is enhanced transmitter/modulator release then it is surprising that the facilitation is eliminated in Ca^{++} free ACSF or nifedipine.

An alternative explanation to account for the dramatic reduction of the facilitation in the presence of the antagonist mixture is that the antagonists effectively shift the concentration-response curve of thapsigargin, cyclopiazonic acid, and A23187 to the right. In the absence of the antagonists, the facilitation due to agonist release might add to the facilitation induced by thapsigargin, cyclopiazonic acid, and A23187. Indeed, there may be a supraditive effect since a rise in intracellular Ca^{++} can shift activation of phospholipase C to the left (Eberhard and Holz, 1988; Rhee *et al.*, 1991). Thus, although part of the facilitation is obviously indirect, there also appears to be a component attributable to a direct action on postsynaptic neurons.

5.4.2 Necessity for external Ca^{++} and rise of internal Ca^{++}

In this study, omission of extracellular Ca^{++} eliminated the NMDA facilitation induced by both 5-HT and thapsigargin. Furthermore, nifedipine blocked the 5-HT, thapsigargin, cyclopiazonic acid, and A23187 mediated facilitation in a concentration-dependent manner. The concentration of nifedipine employed in the present study is in keeping with the concentration necessary to block voltage-dependent (L-type) Ca^{++} channels in pyramidal neurons (Sayer *et al.*, 1992). Taken together, these observations suggest that Ca^{++} influx through voltage-dependent Ca^{++} channels is crucial for the facilitation mediated by 5-HT,

thapsigargin, cyclopiazonic acid and A23187. The α_1 -adrenoceptor mediated facilitation induced by phenylephrine shows similar characteristics (Chapter 7), whereas the facilitation induced by the mGluR agonist 1S,3R-ACPD is insensitive to such treatment (Chapter 7). Thus, the requirement for Ca^{++} entry through voltage-dependent Ca^{++} channels is not necessary for the facilitation *per se*, but instead depends on the drug or agonist used to induce the facilitation.

Presumably the voltage-dependent channels were activated by the NMDA depolarization since all of the present experiments were done in the presence of TTX. Holding the membrane potential constant at the resting membrane potential, as under voltage clamp protocol, would therefore, be expected to abolish the 5-HT, thapsigargin, cyclopiazonic acid, and A23187 induced facilitation. However, 5-HT is effective in facilitating NMDA induced inward currents in cortical neurons under voltage clamp conditions (Reynolds *et al.*, 1988) and the same is true for A23187 at hippocampal neurons (Markram and Segal, 1991). This apparent inconsistency of the present observations with those of Reynolds *et al.* (1988) and Markram and Segal (1991) may reflect: 1) limited space clamp of cortical and hippocampal neurons; 2) that the facilitation requires only low frequency opening of voltage-dependent Ca^{++} channels which might take place at potentials near the resting membrane potential or; 3) that nifedipine blocks a voltage-independent Ca^{++}

channel that is important for the facilitation. In whole cell recording from primary cortical cultures where the space clamp is likely to be better than in slices, 5-HT remains effective in facilitating NMDA currents (Virgo and Neuman, unpublished observations), suggesting that a problem with space clamp may not account for the apparent discrepancy. Clearly, this requires further investigation. Despite the above, nifedipine will be assumed in the following to act on voltage-dependent Ca^{++} channels.

5-HT_{2A} receptor mediated PI hydrolysis is very sensitive to extracellular Ca^{++} (Kendall and Nahorski, 1984; Wojcikiewicz *et al.*, 1993; Yang *et al.*, 1994) which would account for loss of the 5-HT facilitation in the present investigation. The importance of Ca^{++} entry through voltage-dependent Ca^{++} channels to PI hydrolysis has yet to be demonstrated. Alternatively, Ca^{++} entry through voltage-dependent Ca^{++} channels might : 1) amplify signal transduction at phospholipase C (Eberhard and Holz, 1988); 2) modulate Ca^{++} release from IP₃ sensitive and /or ryanodine sensitive Ca^{++} stores (Henzi and MacDermott, 1992) or; 3) add to the rise in intracellular Ca^{++} resulting from NMDA and 5-HT_{2A} receptor activation.

Unlike 5-HT, thapsigargin, cyclopiazonic acid, and A23187 are all capable of raising the concentration of intracellular Ca^{++} in the absence of extracellular Ca^{++} (Pozzan *et al.*, 1994). However, such a rise without a contribution from extracellular Ca^{++} is obviously insufficient to facilitate the NMDA response.

Facilitation of the NMDA depolarization may be triggered by a threshold concentration of intracellular Ca^{++} and several sources may contribute to reaching the threshold.

That a rise in intracellular Ca^{++} is indeed essential for the NMDA facilitation is revealed by the observations made with BAPTA-AM. Perfusing BAPTA-AM eliminated the NMDA facilitation independent of the agent used to induce the facilitation. Presumably by buffering the rise of intracellular Ca^{++} (Niesen *et al.*, 1991), the threshold for inducing the facilitation (see above) could not be reached. Similar observations have been made with respect to the NMDA facilitation in hippocampal neurons induced by carbachol and A23187 (Markram and Segal, 1991, 1992). In these studies, the facilitation was eliminated by the diffusion of BAPTA from the intracellular electrode.

In contrast to a rise in intracellular Ca^{++} facilitating the NMDA depolarization, it has been reported that elevated intracellular Ca^{++} causes a decrease in NMDA receptor activity in cultured hippocampal neurons (Legendre *et al.*, 1993; Vyklicky, 1993) and in cultured spinal neurons (Mayer and Westbrook, 1985, 1987; Mayer *et al.*, 1987). In these studies, intracellular Ca^{++} was increased via entry through the NMDA receptor ionophore or voltage-dependent Ca^{++} channels and thus differs somewhat from the manner in which intracellular Ca^{++} was raised in the present investigation, i.e. in part from release

of Ca^{++} from intracellular organelles. It is thus possible that both an increase and a decrease in the NMDA response could be mediated by a rise in Ca^{++} depending on how the Ca^{++} concentration is elevated and of course on how the change in NMDA response is affected. Differences in tissue preparation, i.e. slice vs cultured neurons (c.f. Markram and Segal, 1991), is unlikely to account for the difference, since 5-HT facilitation has been observed in cultured cortical neurons (Virgo and Neuman, unpublished observations). Further experiments are required to resolve this issue.

In this investigation, removal of extracellular Ca^{++} failed to alter the NMDA depolarization, whereas buffering intracellular Ca^{++} reduced the response. It is likely that in Ca^{++} free medium neuronal membranes are depolarized (Frankenhaeuser and Hodgkin, 1957), and as a consequence, the NMDA response would be enhanced as the inward current produced by NMDA receptor activation would be increased. However, the expected cholinergic and noradrenergic contribution (see Chapter 2) would be reduced in the above medium, i.e. there would be decreased release and the effect of released norepinephrine would be blocked (see Chapter 7). Therefore, the net effect is that the NMDA response remains unchanged in Ca^{++} free medium. On the other hand, the cholinergic and noradrenergic component would be eliminated due to buffering of internal Ca^{++} by BAPTA-AM (see above). This would lead to a reduction of the NMDA response.

5.4.3 Role of calmodulin in loss of the A23187 facilitation

Application of 1 μ M A23187 maximally facilitated the NMDA response, whereas the next highest concentration tested, 3 μ M, reduced the facilitation. Chlorpromazine, but not staurosporine or myo-inositol, prevented the reduction. These results suggest that the rise in intracellular Ca^{++} activates calmodulin (Erondy and Kennedy, 1985) which in turn reduces the facilitation. Calmodulin is reported to be a cytosolic Ca^{++} receptor in neurons (see Gnegy, 1993). Each molecule of this ubiquitous regulatory protein has four Ca^{++} binding sites with dissociation constants in the low micromolar range. When the concentration of Ca^{++} rises into this range, the four binding sites are subsequently occupied and calmodulin becomes a multifunctional activator (Kennedy, 1989). Following activation, calmodulin binds with different proteins and alters their function. One of these proteins, calmodulin dependent kinase II (CaM kinase II), is the predominant Ca^{++} dependent protein kinase in the cortex (Erondy and Kennedy, 1985). This enzyme has relatively wide ranging effects and can phosphorylate several neural proteins (Hanson and Schulman, 1992). Whether reduction of the facilitation is due to phosphorylation of the NMDA receptor complex initiated by CaM kinase II or other calmodulin dependent enzymes remains to be determined. Previously, calmodulin antagonists were found to reduce long term desensitization of the 5-HT_{2A} receptor mediated facilitation (Chapter 3). It remains possible that the

activation of calmodulin induced by A23187 and the 5-HT_{2A} receptor result in similar cellular actions.

5.4.4 Is the facilitation dependent on altering the Mg⁺⁺ dependence of the NMDA receptor?

Reducing the Mg⁺⁺ dependence of the NMDA response (Nowak *et al.*, 1984) could account for the facilitation observed in the present investigation and indeed has been proposed to account for the facilitation of the NMDA response observed in trigeminal neurons as a result of protein kinase C phosphorylation (Chen and Huang, 1992). As expected (Nowak *et al.*, 1984), omission of Mg⁺⁺ from the ACSF results in a substantial increase in the magnitude of the NMDA depolarization of cortical neurons (unpublished observations). Such omission eliminates the 5-HT and phenylephrine induced facilitation, whereas the A23187 facilitation is present, albeit smaller in magnitude. From these observations it is concluded that the facilitation *per se* is not dependent on Mg⁺⁺, i.e., it does not result from altering the voltage-dependent blockade of the NMDA receptor ionophore by Mg⁺⁺ (c.f. Nedergaard *et al.*, 1987).

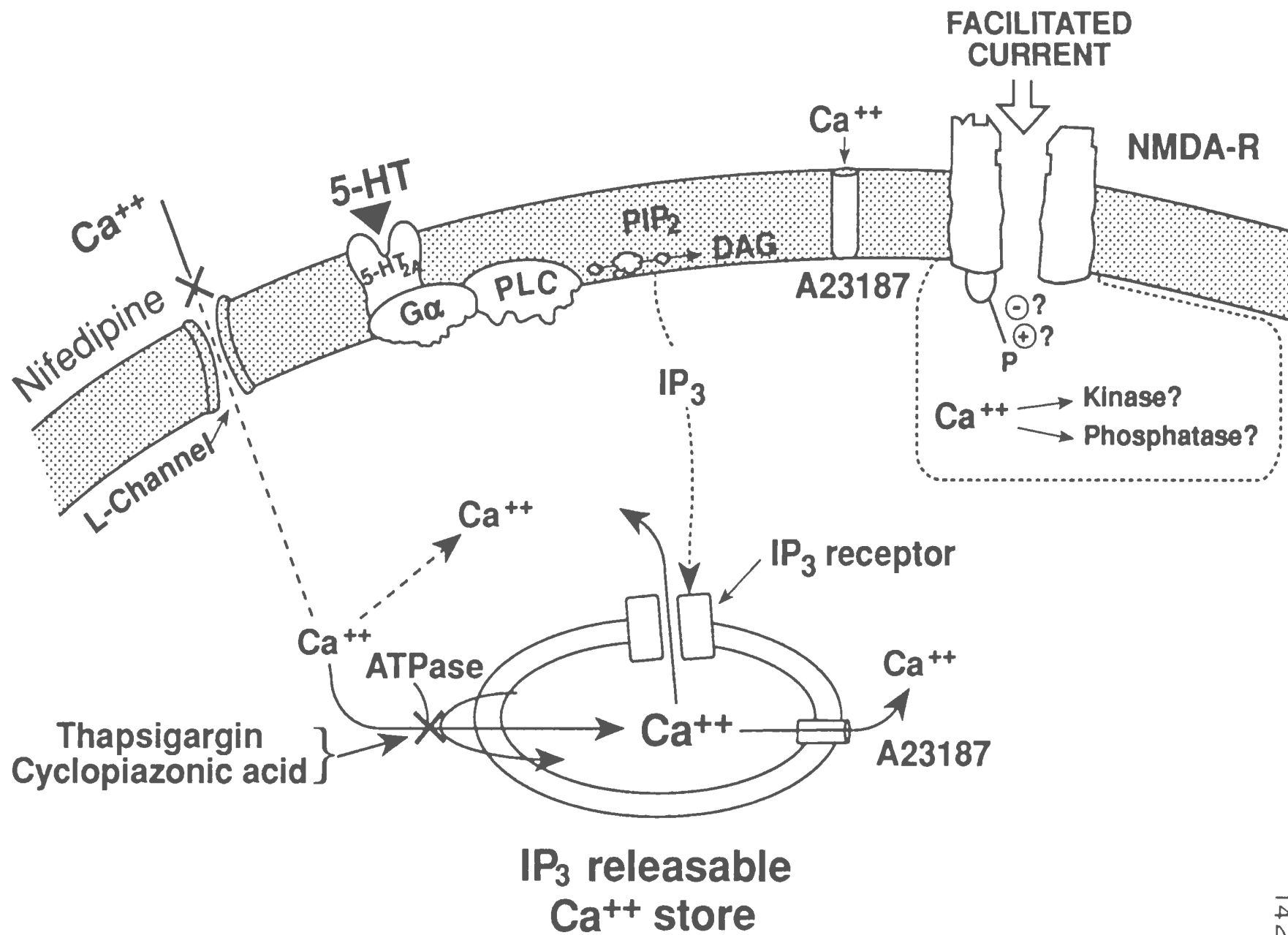
Activation of G-protein complexes does not occur without Mg⁺⁺ (Birnbaumer *et al.*, 1990; Litosh, 1990). Furthermore, removal of extracellular Mg⁺⁺ depresses muscarinic cholinergic mediated responses (Gurwitz and

Sokolovosky, 1980; Aronstam *et al.*, 1985) including muscarinic depolarization of neocortical neurons (El-Behiry and Puil, 1990). Thus, the Mg^{++} dependence of the 5-HT and phenylephrine induced facilitation likely reflects the regulatory role of Mg^{++} on signal transduction of G-protein coupled receptors. Although this is a likely explanation, presumably this occurs following a reduction of the concentration of intracellular Mg^{++} and this remains to be confirmed.

Figure 30 is a cartoon summarizing the results of the present investigation and speculating on how facilitation of the NMDA depolarization may take place at cortical pyramidal neurons. The rise in Ca^{++} is envisioned as being the common mechanism resulting from 5-HT_{2A} receptor activation as well as from the action of thapsigargin, cyclopiazonic acid and A23187, which leads to facilitation of the NMDA depolarization. External Ca^{++} is necessary and enters the cell through voltage-dependent Ca^{++} channels or at the least through a nifedipine sensitive Ca^{++} channel. Ca^{++} is presumed to activate a kinase or phosphatase which regulates the NMDA receptor ionophore by controlling the phosphorylation state of the NMDA receptor or that of a nearby protein, which in turn regulates the NMDA receptor (see Chapter 8 for further discussion on this point). Most importantly, this calcium mediated facilitation of the NMDA receptor is likely to be a common pathway for neurotransmitters regulating NMDA receptors and thus synaptic plasticity in neocortex.

Figure 30. A hypothetical model that illustrates the mode of action by 5-HT, thapsigargin, cyclopiazonic acid and A23187. Although the drugs have different mechanisms of action, they share in common an ability to raise intracellular Ca^{++} that accounts for facilitation of the NMDA depolarization. Abbreviations: 5-HT_{2A} , 5-HT_{2A} receptor; $G\alpha$, α subunit of G-protein; PLC, phospholipase C; PIP_2 , phosphatidyl inositol 4,5-biphosphate; DAG, diacylglycerol; NMDA-R, NMDA receptor.

Figure 3B. A hypothetical model that illustrates the mode of action by 5-HT₂ receptors, cyclo-oxygenase and A12137. Although the drugs have different mechanisms of action, they share in common an ability to raise intracellular Ca²⁺ that accounts for facilitation of the NMDA depolarization. Activation: 5-HT₂ receptor, G_q, a subunit of G-protein, PLC, phospholipase C, PIP₂, inositol 4,5-bisphosphate, DAG, diacylglycerol, NMDA-R, NMDA receptor.



CHAPTER 6

5-HT_{2A} RECEPTOR MEDIATED FACILITATION IN SENESCENT RATS

6.1 Introduction

There is considerable interest in understanding conditions that underlie age related loss in cognitive functions such as memory and learning. Although much of this interest has focused on the forebrain cholinergic system (Barthus *et al.*, 1982), there is a growing awareness that 5-HT may also participate in these processes (Altman and Normile, 1988; Vanderwolf, 1992). Notwithstanding the conflicting literature in this area (Altman and Normile, 1988; Robson *et al.*, 1993), numerous age-related modifications of the central serotonergic system have been reported that could contribute to cognitive deficits. These include changes in fibre morphology, transmitter and metabolite levels, and ligand binding assays for 5-HT_{1B} and 5-HT_{2A} receptors (Battaglia *et al.*, 1987; Gozlan *et al.*, 1990; Marcusson *et al.*, 1984; Robson *et al.*, 1993; van Luijtelaar *et al.*, 1992). A recent report also suggests that coupling between cortical 5-HT_{2A} receptors and G-proteins is reduced with aging (Robson *et al.*, 1993). Age-related change in cortical 5-HT_{2A} receptors may be of particular interest with regard to cognitive deficits. These receptors are concentrated in the frontal cortex (Pazos *et al.*, 1985) and influence the firing

patterns of cortical projection neurons (Neuman and Zebrowska, 1992).

Paradoxically, DOI induced wet dog shakes (a behaviour mediated by 5-HT_{2A} receptors), increases with aging (Gower and Gobert, 1992; Robson *et al.*, 1993). However, the 5-HT_{2A} receptors involved in this response are located in the spinal cord (Fone *et al.*, 1989) and as such, may not reflect modification at cortical sites. At the cellular level, age-related alteration in functional activity of 5-HT_{2A} receptors has not previously been investigated in the frontal cortex. Assessing functional activity is particularly important for G-protein coupled receptors, such as the 5-HT_{2A} receptor. Signal transduction associated with this family of receptors often yields considerable amplification, i.e. the receptors demonstrate receptor reserve (Kenakin, 1993). A reduction in receptor number may not therefore, translate into a significant loss of responsiveness.

As expected from the 5-HT_{2A} receptor/effector system (see Chapters 2 and 3), there are numerous sites at which functional activity may be regulated following ligand binding. With regard to facilitation of the NMDA depolarization induced by 5-HT ligands, the 5-HT component, but not the NMDA component is subject to several forms of desensitization. This desensitization is influenced by protein kinase C activation, availability of the inositol biphosphate precursor myo-inositol, calmodulin activation, and receptor sequestration (Chapters 3 and 4). Age-related processes, aside from reducing the maximum number of 5-HT_{2A} receptors, might

modify cellular or extracellular regulation of these receptors at one or more sites resulting in loss of functional activity. The objective of this study was to examine the 5-HT_{2A} receptor mediated facilitation of the NMDA depolarization in frontal cortex from young adult and senescent rats and compare these findings with the facilitation induced by activating α_1 -adrenoceptors, muscarinic cholinceptors (see Chapter 2) and metabotropic glutamate receptors (see Chapter 7). In addition, this study was an attempt to confirm the evidence that 5-HT_{2A} receptors are located on cortical pyramidal neurons.

6.2 Methods

6.2.1 Electrophysiology

Wedges of sensorimotor cortex from young adult (3-6 months) and senescent (22-34 months) male Fisher 344 rats and young (1-2 months) Sprague-Dawley rats (both from Charles River, Montreal, Quebec) were prepared for recording and analyzed as described in section 2.2.2 and 2.2.5.

6.2.1.1 ACSF composition and drug application

ACSF composition in the bathing medium and drug application were as described in section 2.2.3, 2.2.4, 3.2.2 and 4.2 with the following additions: chelerythrine was perfused for 30 min before 5-HT application, bicuculline, 2-OH saclofen, and tetrodotoxin were perfused throughout. Drug concentrations are given as the salt. Stock solutions were kept frozen until use.

6.2.1.2 Drugs and chemicals

The following drugs were used: chelerythrine (Alomone Labs); 1S,3R ACPD and 3-amino-2-(4-chlorophenyl)-2-hydroxypropyl) sulfonic acid, 2-OH saclofen (Tocris Neuramin); bicuculline (Sigma).

6.2.2 *In situ* hybridization histochemistry

For *in situ* hybridization experiments, Fisher (senescent, 22-30 months, n=3; young adult, 3-6 months, n=3) and Sprague-Dawley rats (3 and 12 months) were used. Rats were anaesthetized with ether then sacrificed by decapitation. The brains were removed and immediately frozen on dry ice. Brain sections, 10 μ m thick, were cut on a cryostat, mounted onto subbed slides and stored frozen until needed.

Before hybridization, sections were brought to room temperature, fixed with 4% paraformaldehyde for 30 min, and rinsed in the following solutions: 1) one rinse in ribonuclease-free H₂O (1 mL diethyl pyrocarbonate/1 L deionized water then autoclaved); 2) 5 min in 0.25 M HCl; 3) 5 min in phosphate buffered saline (PBS); 4) 7.5 min in proteinase K (1 μ g/mL H₂O); 5) 5 min in PBS; 6) 5 min in 0.01M triethanolamine (pH 8.0); 7) 2 X 5 min in PBS. The sections were then incubated in a prehybridization solution (50% formamide, 0.6 M NaCl, 10 mM Tris buffer [pH 7.5], 1 mM ethylenediamine tetraacetic acid, 50 μ g/ml heparin, 10 mM dithiothreitol, 500 μ g/ml tRNA, 0.2% Tween 20, 100 μ g/mL salmon sperm DNA) for one hour in a humid chamber at 42°C. Probes were 3' end-labelled with [³⁵S] d-ATP (Dupont Canada, NEN Research Products) to a specific activity ranging between 2.4×10^6 and 6.7×10^6 cpm/pmol using a terminal transferase kit (Dupont Canada). Sections were incubated at 42°C for 18-22 hours in 100 μ L of

hybridization solution containing the radiolabelled oligonucleotide (2×10^6 cpm/slide). Sections were rinsed in 2X sodium chloride/sodium citrate (SSC) at room temperature, 2X SSC at 55°C, 0.2X SSC at 55°C and then air dried for 2-3 hrs before dipping in emulsion.

Slides were dipped in autoradiographic emulsion (Kodak NTB-2) mixed 1:1 with 600 mM ammonium acetate (42-45°C), dried for at least 2 hrs and stored in the dark at 4°C for 10 days, three weeks or five weeks then developed in Kodak D-19 (2 min), rinsed in H₂O (30 sec) and fixed with Kodak fixer (4 min). The sections were washed in H₂O, counterstained with cresyl violet, dehydrated, coverslipped with Permount and viewed under bright- and darkfield microscopy.

The antisense probe used to detect mRNA for the 5-HT₂ receptor was a commercially available (Dupont Canada) mixture of three oligonucleotides complementary to the bases 3-51, 721-770 and 811-858 of the 5-HT₂ receptor mRNA (Pritchett *et al.*, 1988). A computer search revealed these sequences were unique for the 5-HT_{2A} receptor. Combining ³⁵S-labelled and unlabelled oligonucleotide in a 1:10 ratio and using the resulting mixture for hybridization served as a control. This control was employed on slides containing sections adjacent to those exposed to antisense probes.

6.2.2.1 Quantitative analysis

Silver grains over cells were quantitated using bright and dark field microscopy (X40 objective) coupled to an image analysis system (Bioquant, R & M Biometrics). Pyramidal and non-pyramidal neurons in layer V were studied by analyzing every identifiable cell in a digitized image field. Image enhancement processing was used to facilitate the visualization and identification of cells under bright field illumination. Only those cells with a visualized nucleus were analyzed and each cell was identified on the basis of position, shape, Nissl staining intensity and size. The borders of identified cells were outlined using a cursor coupled to the computer and a graphics table (Summasketch II, Summagraphics). Using darkfield illumination the number of silver grains within the border of each soma was determined and normalized to grains/100 μm^2 . Four to five fields/section were used for statistical analysis. The background density of silver grains was determined from regions that lacked cell bodies. Analysis of variance and the post hoc Tukey-Kramer multiple comparisons test were used to analyze the results.

6.3 Results

6.3.1 Electrophysiology

In keeping with previous observations (Chapter 2), co-perfusion of 30 μM 5-HT and NMDA for 2 min reversibly enhanced the NMDA depolarization in wedges prepared from young adult Fisher 344 rats (Fig. 31). In contrast, 30 μM 5-HT did not facilitate the NMDA depolarization in wedges from senescent animals (Fig. 31). Concentration response data for the 5-HT facilitation in young adult and senescent rats are shown in Fig. 32. Amplitude of the NMDA depolarization was smaller in senescent rats compared to young adults, but this difference did not reach statistical significance (0.51 ± 0.07 mV, $n=17$, vs. 0.75 ± 0.11 mV, $n=17$; $p=0.075$, two tailed t test).

Application of 5-HT presumably activates all 5-HT receptor subtypes present in the cortical wedge. At least one of these receptors, the 5-HT_{1A} subtype, reduces the amplitude of the NMDA depolarization (Chapter 2). DOI, an agonist at 5-HT_{2A} and 5-HT_{2C} receptors, was substituted for 5-HT to ascertain whether the absence of facilitation reflected a loss of 5-HT_{2A} receptor signal transduction or an enhanced contribution from an inhibitory 5-HT receptor. Although DOI facilitated the NMDA depolarization in wedges from young adult rats, no such facilitation was observed in wedges prepared from senescent rats (Fig. 33).

Agonists at other receptors that activate phospholipase C, including carbachol, 1S,3R ACPD (Chapter 7), and phenylephrine also facilitate the NMDA depolarization in cortical wedges (Chapter 2). As expected, 10 μ M carbachol, 50 μ M 1S,3R ACPD, and 10 μ M phenylephrine significantly enhanced the NMDA depolarization in wedges from young adult rats. However, in contrast to 5-HT, the facilitation induced by these agonists in wedges from senescent rats was not significantly different from that in the younger animals (Fig. 33).

Receptor binding assays suggest that 5-HT_{2A} receptors are present on cortical neurons in senescent rats (Battaglia *et al.*, 1987; Gozlan *et al.*, 1990; Robson *et al.*, 1993). The absence of functional activity could then represent some form of receptor desensitization. Activating protein kinase C with phorbol ester or oleoacetyl glycerol is particularly effective in eliminating the 5-HT facilitation, an action inhibited by staurosporine (Chapter 3). When wedges from senescent animals were perfused with 10 nM staurosporine for 40 min, 30 μ M 5-HT induced a significant facilitation of the NMDA depolarization (Fig. 34A). Amplitude of the resulting facilitation was such that it did not differ significantly from that observed in wedges from young adult rats in the presence of staurosporine (Fig. 34B).

Staurosporine exhibits only marginal selectivity for protein kinase C in comparison to other kinases (Ruegg and Burgess, 1989). Chelerythrine (1 μ M), a specific protein kinase C inhibitor (Herbert *et al.* 1990), also restored the 5-HT

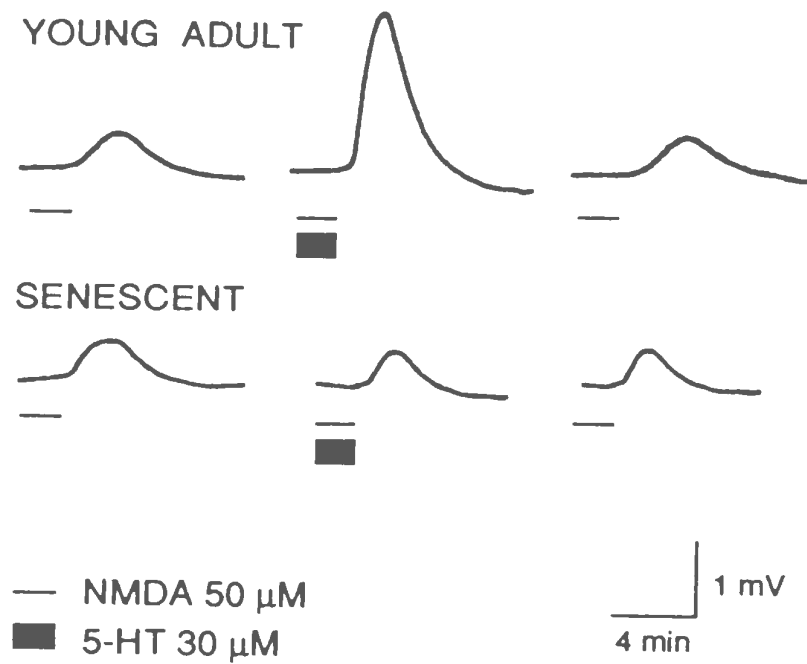


Figure 31. 5-HT facilitates NMDA depolarization of cortical neurons in young adult, but not in senescent rats. Cortical wedges from young adult and senescent rats, upper and lower panel respectively. Control response to 50 μM NMDA (left). Perfusion of 30 μM 5-HT and NMDA (centre). Recovery 20 min later (right). Upward deflection is negative.

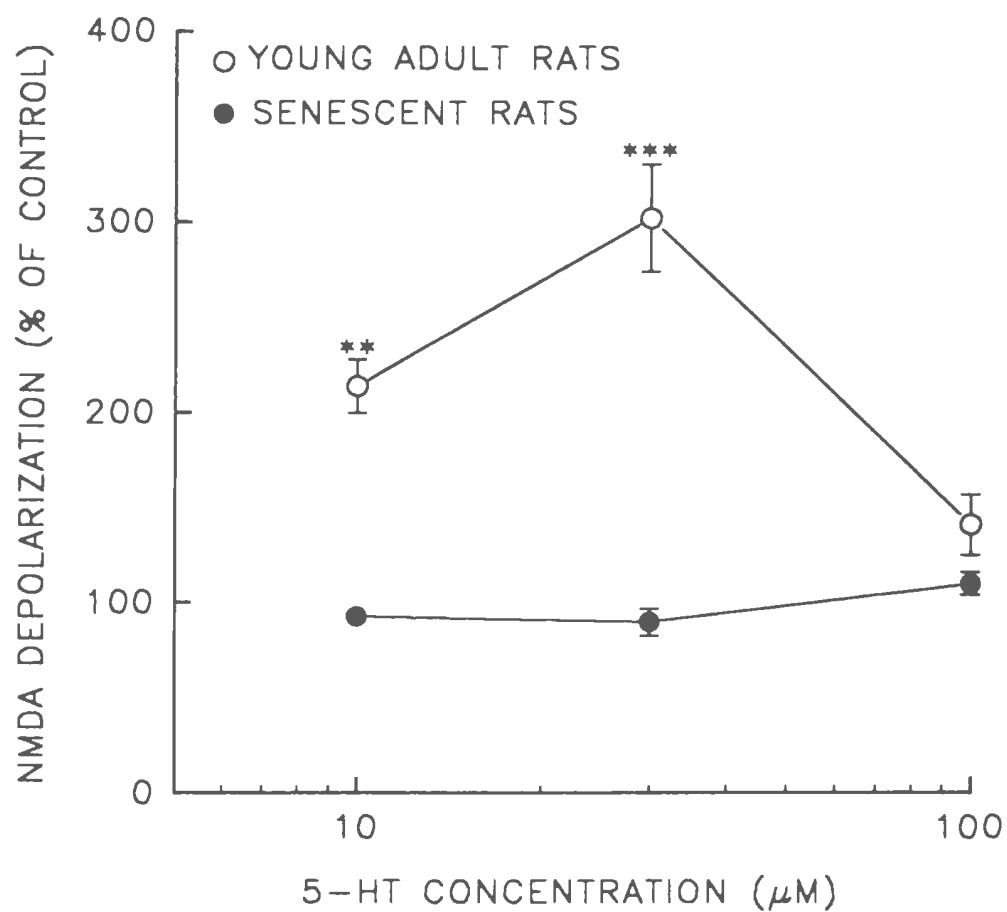


Figure 32. Concentration-response relationship for 5-HT facilitation of NMDA depolarization in young adult and senescent rats. NMDA concentration was 50 μM. 5 to 8 wedges were used for each concentration. ***, $p < 0.001$.

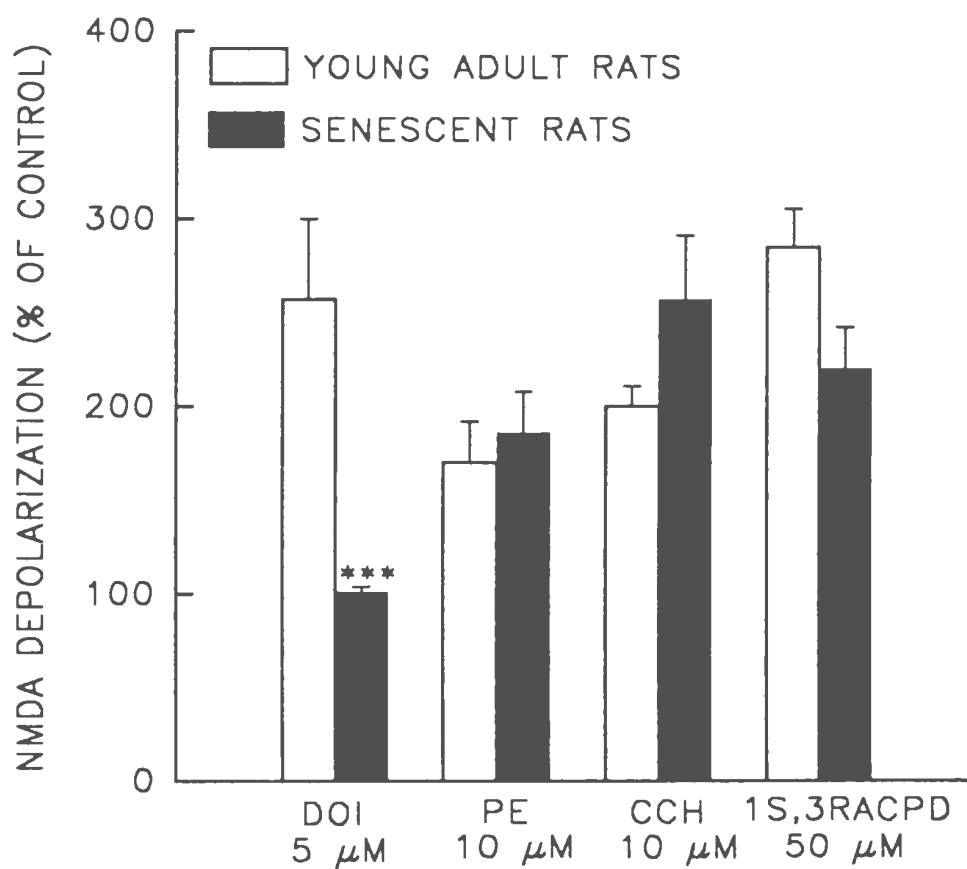


Figure 33. Differential action of DOI, carbachol and phenylephrine and 1S,3R-ACPD. DOI facilitates the NMDA depolarization only in the young adult group, whereas carbachol, phenylephrine and 1S,3R-ACPD facilitate the NMDA depolarization in both groups. 5 to 6 wedges were used for each measurement. ***, $p < 0.001$.

facilitation in 4 wedges from 2 senescent rats (Fig. 34B). In a further 2 wedges from a 34 month old rat, chelerythrine failed to alter the NMDA response.

Concanavalin A, calmodulin antagonists, and myo-inositol also reduce desensitization of the 5-HT_{2A} receptor/effector system (Chapter 3 and 4). Perfusing wedges with 100 nM concanavalin A, 1 μ M calmidazolium or 1 μ M chlorpromazine failed to reestablish the 5-HT facilitation in senescent rats despite some potentiation of the facilitation in young adult rats (Table III). As expected (Chapter 4), perfusion with 10 mM myo-inositol potentiated the facilitation in wedges from young adult rats (Fig. 35). However, in wedges from senescent rats myo-inositol did not restore the facilitation (Fig. 35). It has been suggested that 5-HT_{2A} receptors in the frontal cortex are located on GABAergic interneurons (Mengod *et al.*, 1990; Morilak *et al.*, 1993). However, as shown in Fig. 36, application of 30 μ M 5-HT in the presence of 10 μ M bicuculline, 10 μ M 2-OH saclofen (blockers of GABA_A and GABA_B receptors respectively) and 0.2 μ M tetrodotoxin (TTX; to prevent paroxysmal depolarizing shifts) resulted in facilitation of the NMDA depolarization ($215 \pm 22\%$, n=4). The combination of bicuculline, 2-OH saclofen and TTX did not alter the NMDA depolarization ($98 \pm 11\%$, n=4).

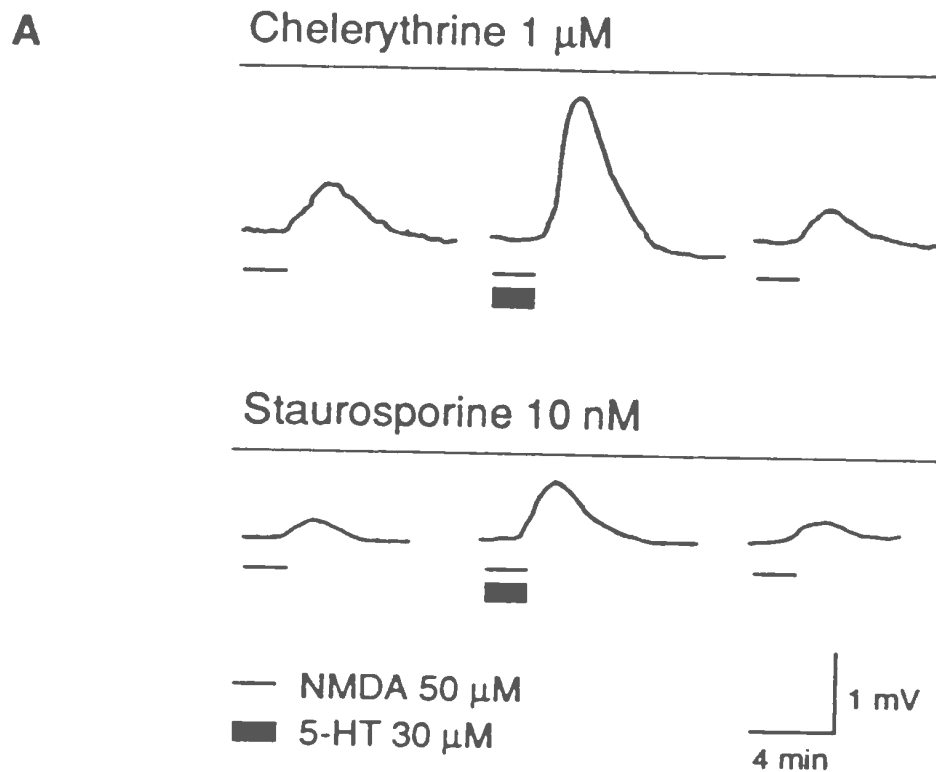


Figure 34. Staurosporine and chelerythrine restore the 5-HT facilitation in senescent rats. **A:** tracings showing 5-HT facilitates the NMDA depolarization in wedges from senescent rats during perfusion with 1 μ M chelerythrine (upper trace) or 10 nM staurosporine (lower trace). **B:** averaged data for staurosporine in young adult and senescent rats and for chelerythrine in senescent rats. 5 to 6 wedges were used for each measurement. ***, $p < 0.001$, staurosporine and chelerythrine vs. senescent control.

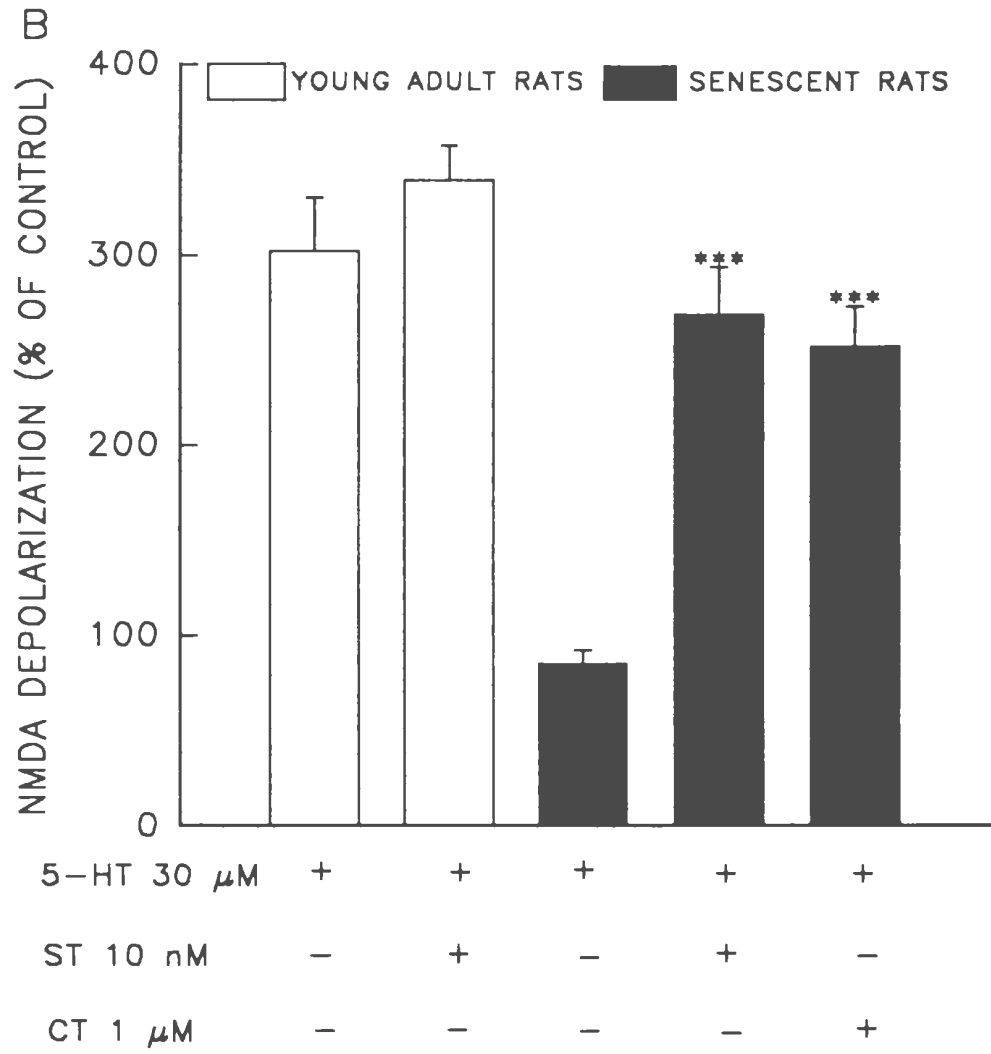


TABLE III. Effects of Concanavalin A, calmidazolium and chlorpromazine on 5-HT facilitation

DRUG	NMDA depolarization (% of control)	
	Senescent	Young adult
5-HT 100 μ M	110 \pm 6% (n=6)	141 \pm 16% (n=5)
+ Concanavalin A 100 nM	138 \pm 18% (n=7)	380 \pm 43% (n=4)***
+ Calmidazolium 1 μ M	128 \pm 10% (n=5)	204 \pm 13% (n=5)
+ Chlorpromazine 1 μ M	138 \pm 8.5% (n=4)	275 \pm 38% (n=4)*

Concanavalin A prevents receptor sequestration (Lin and Levitan, 1991; Chapter 3), whereas calmidazolium and chlorpromazine are calmodulin antagonists (Chupran *et al.*, 1991; Masson *et al.*, 1992). Statistical comparisons were by one way analysis of variance followed by Bonferroni test. All treatments were compared with 5-HT 100 μ M (control). ***, $p < 0.001$, 5-HT vs. 5-HT + concanavalin A. *, $p < 0.05$, 5-HT vs 5-HT + chlorpromazine.

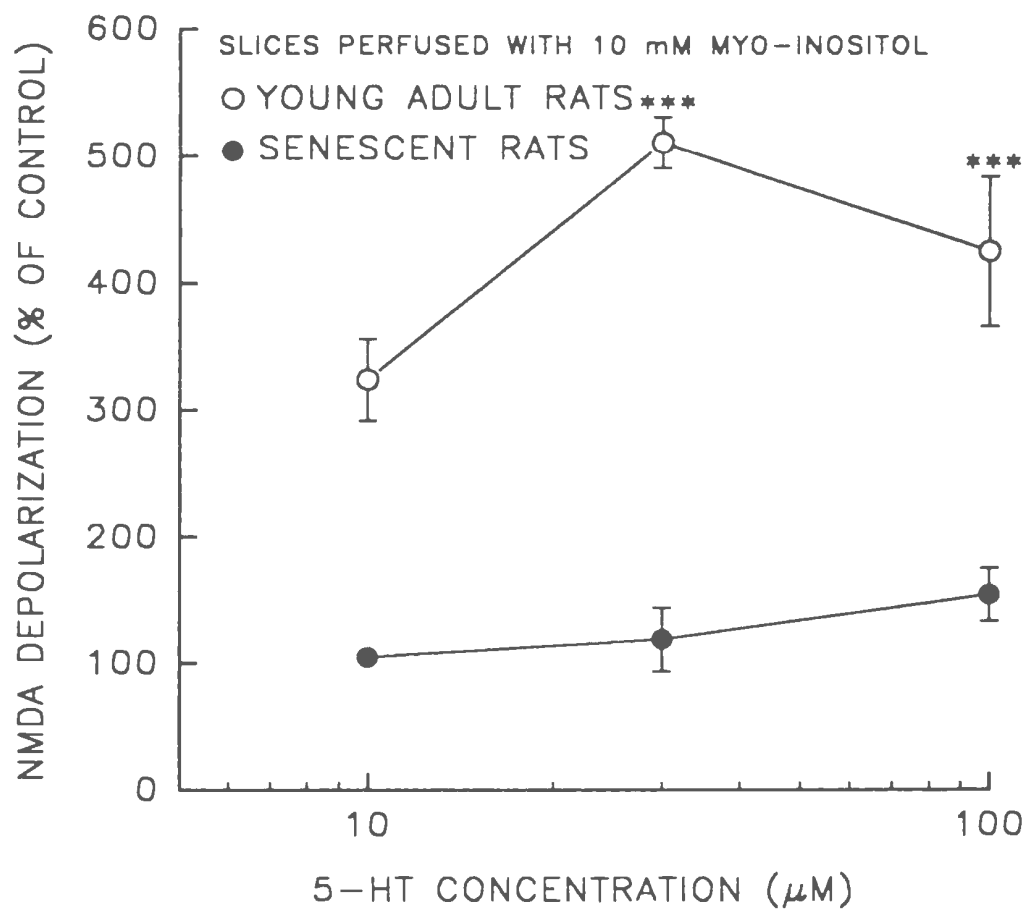


Figure 35. Concentration-response relationship for 5-HT facilitation in young adult and senescent rats in the presence of 10 mM myo-inositol. 5 to 8 wedges were used for each point. ***, $p < 0.001$.

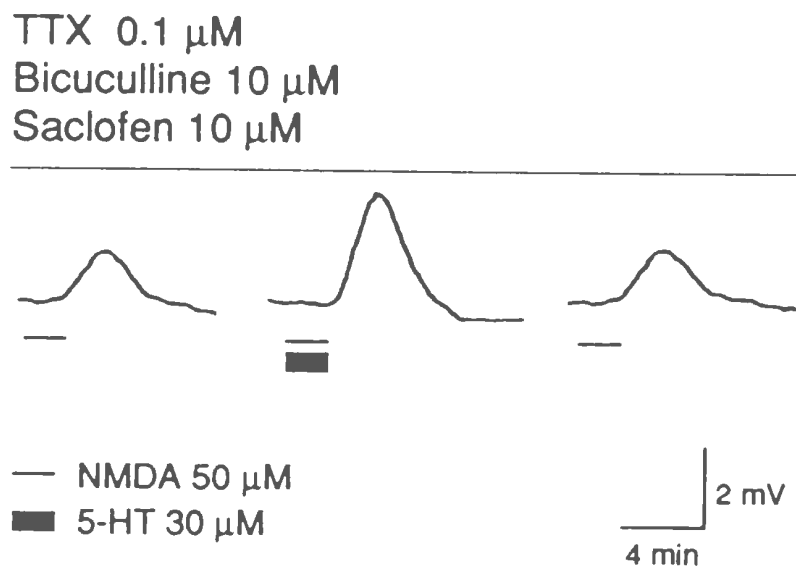


Figure 36. GABA_A and GABA_B antagonists do not eliminate the 5-HT facilitation. Traces from a cortical wedge prepared from a young Sprague-Dawley rat perfused with mixture as shown. TTX was added to prevent paroxysmal depolarizing shifts in the presence of GABA receptor antagonism.

6.3.2 *In situ* hybridization

Localization of cortical 5-HT_{2A} receptors was investigated using a labelled probe for 5-HT_{2A} receptor mRNA. *In situ* hybridization in both young adult and senescent rats resulted in cell labelling in cortical layers II to VI of the sensorimotor cortex with most labelled cells located in layers II and V (Fig. 37). On the other hand, mixing labelled and unlabelled probe (1:10) resulted in background loss of label (data not shown). Visualization of cells labelled by the antisense probe revealed that only a subset of pyramidal cells were labelled (Fig. 38). Further quantitative analysis of silver grains was made in layer V, the area containing the highest density of label in senescent and young adult animals. Within animal statistical analysis revealed a significantly greater density of grains over pyramidal cells than background regions, whereas non-pyramidal cells did not differ from background labelling (Table IV). The analysis also revealed that the density of grains present over pyramidal cells was significantly greater than over nonpyramidal cells (Table IV).

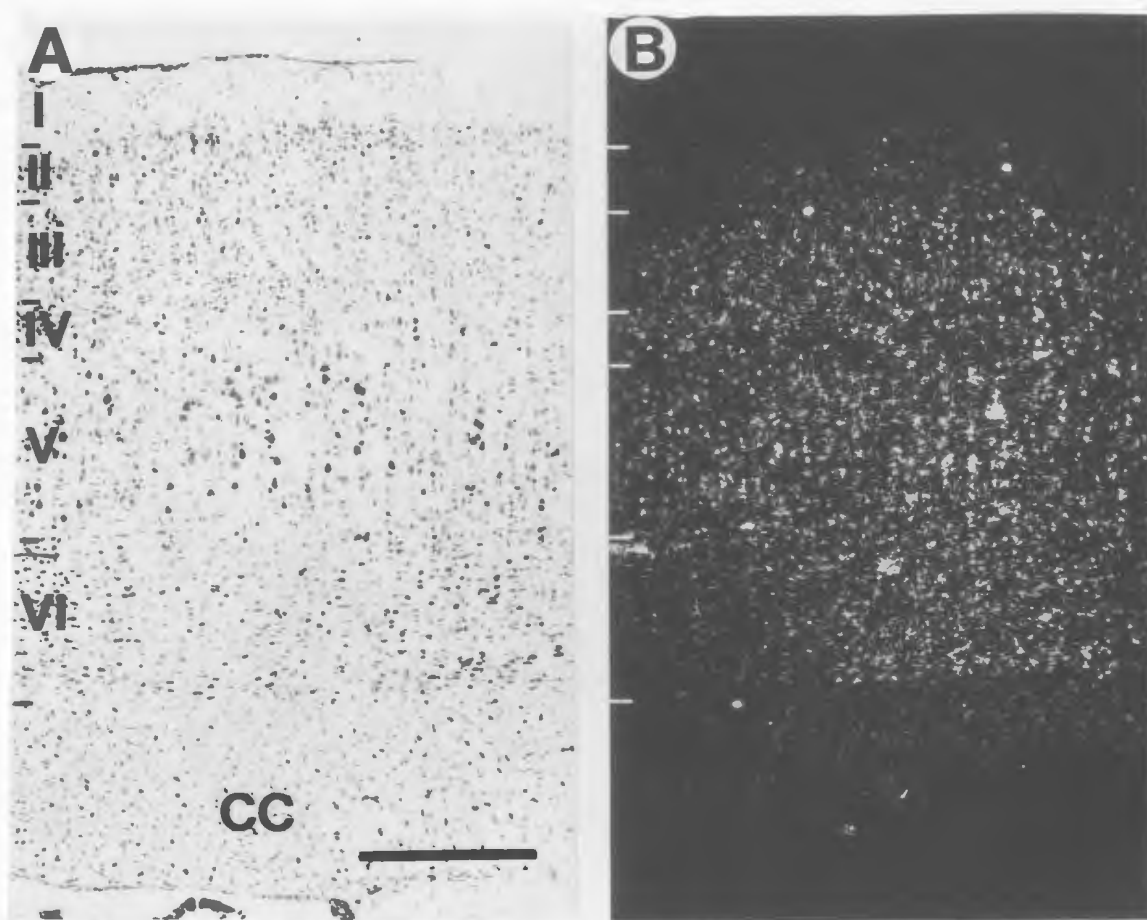


Figure 37. Bright (A) and darkfield (B) photomicrographs of the frontal/parietal cortex showing the distribution of silver grains after *in situ* hybridization histochemistry with ^{35}S -labelled antisense probe for $5\text{-HT}_{2\text{A}}$ receptor mRNA in the various layers of adult Sprague-Dawley rat cortex. Sagittal section. Scale bar is $500\ \mu\text{M}$.

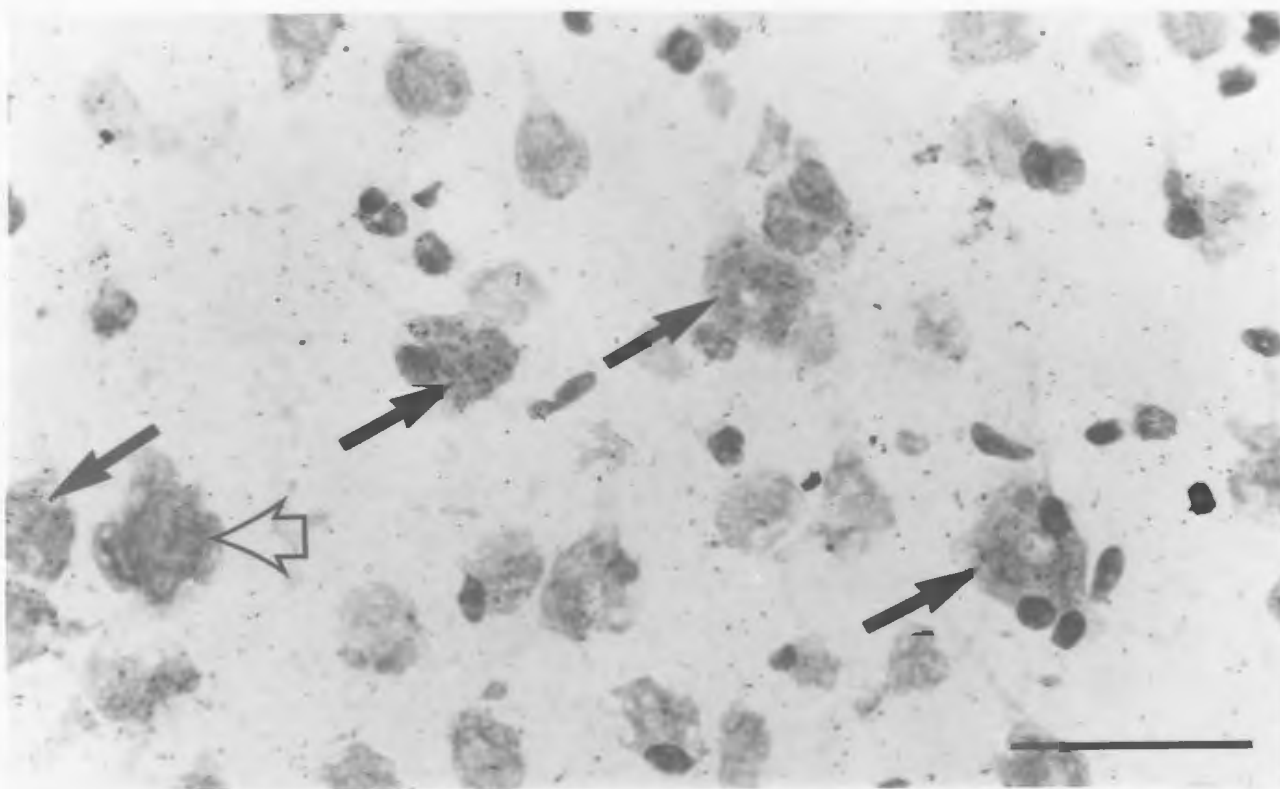


Figure 38. Bright field photomicrograph (high magnification) of the frontal/parietal cortex showing localization of 5-HT_{2A} mRNA in layer V of senescent rat (26 months old). Solid arrows point to labelled pyramidal cells. The open arrow points to a nonlabelled pyramidal cell. Scale bar is 50 μ M.

TABLE IV. Density of grains (Mean \pm SEM) over different cell types or background region. The number of cells analyzed is indicated in parentheses.

Animal	Cell type (grains/ 100 μm^2)		
	Background	Pyramidal	Non-pyramidal
Young adult,			
Fisher	3.4 \pm 0.5 (n=6)	9.2 \pm 1.0 (n=6)**	5.6 \pm 1.1 (n=8)'
Young adult, S.D.	2.4 \pm 0.2 (n=5)	6.8 \pm 0.9 (n=9)*	3.6 \pm 0.9(n=10)
26 month, Fisher	6.9 \pm 0.7 (n=5)	14.7 \pm 2.2 (n=7)*	7.7 \pm 1.0 (n=6)'
30 month, Fisher	3.4 \pm 0.2 (n=5)	6.6 \pm 0.7 (n=11)*	3.7 \pm 0.7(n=13)"

Fisher, Fisher 344 rat. S.D., Sprague-Dawley rat. All within animal one way ANOVAs indicate significant differences between the groups. In all cases, post hoc Tukey tests show that the density of grains over pyramidal cells is significantly different from controls and non-pyramidal cells while that over non-pyramidal cells is not different from background. *, $p < 0.05$, pyramidal cells vs background or non-pyramidal cells; **, $p < 0.01$, pyramidal cells vs background or non-pyramidal cells. ', $p < 0.05$, non-pyramidal cells vs pyramidal cells; ", $p < 0.01$, non-pyramidal cells vs pyramidal cells.

6.4 Discussion

The major findings from this investigation are: 1) the presence of 5-HT_{2A} receptor mRNA in pyramidal neurons from both young adult and senescent rats; 2) selective loss of 5-HT_{2A} signal transduction with senescence; 3) restoration of the 5-HT_{2A} facilitation following inhibition of protein kinase C.

In interpreting the results of this study it should be noted that only young adult (6 months) and senescent rats (22 to 34 months) were compared. None of the wedges from senescent rats tested showed signs of facilitation, unless perfused with a protein kinase C inhibitor. Had animals at intermediate ages been tested, evidence for a gradual loss of function might have been obtained. Accordingly, when reference is made to loss of function in senescent rats, it should be understood that this loss could be complete at ages significantly younger than those tested.

6.4.1 5-HT_{2A} receptors are located on pyramidal neurons

An important consideration in understanding the apparent loss of 5-HT_{2A} receptor mediated signal transduction is the cellular localization (frontal cortex) of the 5-HT_{2A} receptors which mediate facilitation of the NMDA depolarization. Grease-gap recording only registers depolarization of neurons whose axons pass through the grease seal. As discussed (see Chapter 2), projection neurons meet this

requirement (pyramidal neurons in layers II/III and V and some neurons in layer VI; Lorente De No, 1949), whereas interneurons do not. This does not, however, preclude tonic or phasic transmitter/modulator release from nerve terminals or interneurons contributing to the depolarization of projection neurons, and consequently, having an influence on the potential recorded (see Chapter 2). Thus, depending on whether 5-HT acts directly or indirectly on projection neurons, the deficit observed in cortical wedges from senescent rats might reflect i) loss of 5-HT_{2A} receptor associated signal transduction or ii) loss of effectiveness of the indirectly acting agent.

The presence of silver grains over pyramidal neurons, but not interneurons, of the frontal cortex following *in situ* hybridization with ³⁵S-labelled probe for 5-HT_{2A} receptor mRNA suggests that 5-HT_{2A} receptors are present on projection neurons and that 5-HT does indeed act directly. This should not be taken to preclude the possibility that functional 5-HT_{2A} receptors do not exist on interneurons or perhaps, glial cells. The presence of 5-HT_{2A} mRNA in human pyramidal neurons has also been observed by Burnet (P.W.J. Burnet, Department of Neuropathology, Oxford University, personal communication). The commercial probe utilized in this study contains three antisense oligonucleotide sequences for the 5-HT_{2A} receptor, thereby greatly increasing the probability of detection. Of course, the presence of a particular mRNA is not in itself proof that the protein is

expressed. However, the presence of 5-HT_{2A} receptor mRNA on projection neurons is consistent with the high density of 5-HT_{2A} binding sites in layers III and V (Pazos *et al.*, 1985) and with the electrophysiological and the pharmacological evidence for a direct action of 5-HT on layer V pyramidal neurons (see Chapter 2).

In piriform cortex, electrophysiological (Sheldon and Aghajanian, 1990) and immunohistochemical (Morilak *et al.*, 1993) evidence indicates the presence of 5-HT_{2A} receptors on GABAergic interneurons. Activation of these receptors leads to the appearance of inhibitory post synaptic potentials on pyramidal neurons in layer II of piriform cortex (Sheldon and Aghajanian, 1990). In contrast, recordings from pyramidal neurons in frontal and cingulate cortex do not reveal either inhibitory- or excitatory post synaptic potentials following application of 5-HT (Araneda and Andrade, 1991; Davis *et al.*, 1987; Nedergaard *et al.*, 1987; Reynolds *et al.*, 1988; Tanaka and North, 1993). Cellular responses attributable to 5-HT_{2A} receptor activation on pyramidal neurons, including membrane depolarization (Araneda and Andrade, 1991; Davies *et al.*, 1987; Tanaka and North, 1993) and facilitation of the NMDA depolarization (Nedergaard *et al.*, 1988; Reynolds *et al.*, 1988; Chapter 2) are not blocked by TTX, consistent with 5-HT having a direct action. Were 5-HT to act indirectly through a GABAergic mechanism (Mengod *et al.*, 1990; Morilak *et al.*, 1993), the mixture of GABA_A and GABA_B receptor

antagonists should have eliminated the 5-HT facilitation. The smaller amplitude of the facilitation observed during perfusion of this mixture can be attributed to TTX, which alone reduces the amplitude of the 5-HT facilitation (Chapter 2) and decreases phosphoinositide hydrolysis (Gurwitz and Sokolovsky, 1987).

Further evidence for a direct action by 5-HT comes from studies on heterologous desensitization of muscarinic cholinceptors induced by prior application of 5-HT (Chapters 3 and 4). This heterologous desensitization results from depleting substrate for phospholipase C, since it is prevented by perfusing myo-inositol (Chapter 4), the substrate precursor. The presence of substrate dependent heterologous desensitization and its reversal by myo-inositol argues strongly that muscarinic cholinceptors and 5-HT_{2A} receptors are co-localized on the same neurons. The finding that muscarinic cholinceptor stimulation facilitates the NMDA depolarization (Chapter 2) and that muscarinic cholinceptors are present in abundance on pyramidal neurons (Van der Zee *et al.*, 1993) provides compelling evidence for the presence of 5-HT_{2A} receptors on pyramidal neurons.

From the above it is concluded that 5-HT_{2A} receptors are located on projection neurons and, as a consequence, 5-HT acts directly to facilitate the NMDA depolarization. These results are not easily reconciled with a recent report on localizing 5-HT_{2A} receptors using antibodies raised against a sequence of the 5-HT_{2A} receptor (Morilak *et al.*, 1993). The presence of antibody labelling in regions

such as the piriform cortex is consistent with electrophysiological evidence (Sheldon and Aghajanian, 1990) but, as discussed above, failure to observe label on projection neurons is not. Moreover, autoradiography reveals extensive 5-HT_{2A} ligand binding in the rat frontal cortex (Gozlan *et al.*, 1990; Pazos *et al.*, 1985) whereas 5-HT_{2A} immunoreactive neurons in the same area are rather sparse (Morilak *et al.*, 1993). These discrepancies remain to be clarified.

6.4.2 Mechanism(s) underlying loss of 5-HT_{2A} receptor activity

The cellular mechanism(s) responsible for facilitation of the NMDA depolarization of cortical neurons is (are) unknown, although the involvement of PKC and calmodulin have been excluded (Chapters 3 and 4). However, a rise in intracellular Ca⁺⁺ is necessary (Chapter 5). Buffering intracellular Ca⁺⁺ eliminates agonist induced facilitation, whereas agents which raise intracellular Ca⁺⁺, such as thapsigargin and cyclopiazonic acid, facilitate the NMDA response (Chapter 5). This commonality in mechanism, along with the present observation that the facilitation induced by carbachol, phenylephrine and 1S,3R ACPD is unchanged in wedges from senescent rats, implies that the loss observed with 5-HT and DOI relates to a deficit in signal transduction associated with the 5-HT_{2A} receptor and not with a process distal to this stage, e.g. at the NMDA receptor. Preliminary investigation (Neuman and Zhang, unpublished observations) indicates that the

deficit in 5-HT_{2A} signal transduction is also present *in situ*. Accordingly, loss of the 5-HT_{2A} response observed *in vitro* cannot, for example, be attributed simply to damage sustained during wedge preparation.

Consistent with the age-related decline in NMDA receptor binding in the cortex (Tamaru *et al.*, 1991), a smaller though not statistically significant NMDA depolarization was observed in wedges from senescent rats. When the 5-HT concentration is held constant, the facilitation varies with NMDA concentration (Chapter 2). Accordingly, the smaller NMDA depolarization could contribute to loss of the facilitation. However, the facilitation restored during perfusion of the protein kinase C inhibitors did not differ significantly between wedges prepared from young adult and senescent rats, making it unlikely a decline of the NMDA response contributes significantly to the deficit.

Whether 5-HT_{2A} receptors are functional or not, they are present on cortical neurons in senescent rats judging from ligand binding studies (Battaglia *et al.*, 1987; Gozlan *et al.*, 1990; Robson *et al.*, 1993). Loss of 5-HT_{2A} receptor signal transduction could then simply reflect receptor desensitization. This desensitization does not involve agonist induced receptor internalization, calmodulin dependent long term desensitization, or substrate depletion to judge from the failure of concanavalin A, calmodulin antagonists, and myo-inositol respectively, to reestablish the facilitation (Chapters 3 and 4). On the other hand, restoration of the

facilitation during perfusion with staurosporine and chelerythrine clearly implicates a major role for protein kinase C in accounting for the loss of signal transduction. Phorbol ester or oleoacetyl glycerol induced activation of protein kinase C does not alter the NMDA evoked depolarization of cortical neurons, whereas it does eliminate the 5-HT facilitation (Chapter 3). Stimulation of 5-HT_{2A} receptors with 5-HT or DOI also leads to activation of protein kinase C, which in turn inhibits 5-HT_{2A} signal transduction (Aghajanian, 1990; Chapter 3). Feedback inhibition by protein kinase C is in keeping with the presence of protein kinase C consensus sequences on the 5-HT_{2A} receptor (Roth *et al.*, 1990). Staurosporine, although a potent inhibitor of protein kinase C ($IC_{50}=2.7$ nM; Tamaoki *et al.*, 1986), is far from being selective (Ruegg and Burgess, 1989). Chelerythrine, on the other hand, has an IC_{50} for protein kinase C (0.66 μ M) which is over two orders of magnitude lower than its IC_{50} for other protein kinases (Herbert *et al.*, 1990). Given the selectivity of chelerythrine and supportive data with staurosporine, it is suggested that inhibition of protein kinase C accounts for restoration of the 5-HT facilitation. The lack of response to chelerythrine in the oldest rat tested, 34 months, may indicate an additional age-related decline in signal transduction unrelated to protein kinase C.

Selective activation of protein kinase C could arise as a result of excessive 5-HT release in wedges prepared from senescent rats. Turnover of cortical 5-HT

increases in senescent rats (Gozlan *et al.*, 1990; Robson *et al.*, 1993; van Luijtelaar *et al.*, 1992), perhaps as a consequence of declining 5-HT_{1B} autoreceptors (Gozlan *et al.*, 1990; Murphy and Zelman, 1989), or degenerating 5-HT containing fibres (van Luijtelaar *et al.*, 1992). Chronic overstimulation of 5-HT_{2A} receptors could activate protein kinase C resulting in feedback inhibition sufficient to reduce the number of operative 5-HT_{2A} receptors below the threshold necessary to initiate facilitation. Phosphorylation of the 5-HT_{2A} receptor might account for the suggested uncoupling of the receptor from G-proteins (Robson *et al.*, 1993). In this model the receptors are immediately available for stimulation once the inhibitory feedback is removed, i.e., once protein kinase C is blocked. Depending on the level of 5-HT released, limited receptor internalization could take place, accounting for the decline in 5-HT_{2A} binding observed in some studies (Battaglia *et al.*, 1987; Gozlan *et al.*, 1990).

An alternative suggestion compatible with the present observations is that the inhibition by protein kinase C, feed-forward inhibition in this case, results from heterologous regulation by another transmitter/modulator that activates protein kinase C. Unlike homologous overstimulation (*vide supra*), this model must account for selectivity with respect to the protein kinase C mediated loss of the 5-HT facilitation. However, preliminary observations reveal that cortical muscarinic cholinergic receptors (Kong and Neuman, personal communication), metabotropic

glutamate receptors (Chapter 7), and α_1 -adrenoceptors show little or no sensitivity to activation of protein kinase C by phorbol diacetate, at least with respect to their ability to facilitate NMDA depolarization. Thus altered activation of one of these receptors, their associated second messenger cascade, or indeed another receptor which stimulates protein kinase C could account for selective loss of the 5-HT_{2A} receptor mediated facilitation without reducing the ability of other modulators (see above) to facilitate the NMDA response. Of course, it remains to be demonstrated that activation of a receptor that stimulates protein kinase C heterologously regulates activity of 5-HT_{2A} receptors.

In conclusion, the experiments in this study strongly support the hypothesis that 5-HT_{2A} receptors are expressed in cortical pyramidal neurons and that 5-HT facilitation of the NMDA response is a direct consequence of receptor activation. Furthermore, signal transduction mediated by 5-HT_{2A} receptors may be significantly impaired with aging.

CHAPTER 7

METABOTROPIC GLUTAMATE RECEPTORS MEDIATE FACILITATION OF THE NMDA DEPOLARIZATION: A COMPARISON WITH THE 5-HT_{2A} RECEPTOR

7.1 Introduction

As demonstrated in the preceding studies (Chapters 2, 3 and 4), projection neurons (mainly pyramidal cells; Lorente de No, 1949) of the cerebral cortex are depolarized by NMDA and this depolarization is facilitated following activation of 5-HT_{2A} receptors. 5-HT_{2A} induced facilitation likely occurs through a mechanism dependent on activating PLC-PI and the resulting second messenger cascade. This is consistent with the evidence suggesting that activation of G-protein coupled receptors, which utilize the PLC-PI pathway for their signal transduction mechanism, facilitate responses to NMDA in hippocampus (Markram and Segal, 1992). If the PLC-PI response provides a common pathway for enhancement of the NMDA depolarization then metabotropic glutamate receptors, which stimulate the PLC-PI pathway, should behave in a similar manner.

The metabotropic glutamate receptor (mGluR) is another member of the G-protein receptor superfamily that contains receptor subtypes which couple positively to phospholipase C (Masu *et al.*, 1993; Bockaert and Fagni, 1993). Thus

mGluR1 and the mGluR5 have been shown to activate phospholipase C (Abe *et al.*, 1992; Tanabe *et al.*, 1992). These receptors differ in regional abundance and pharmacological characteristics (Abe *et al.*, 1992; Schoepp and Conn, 1993; Shigemoto *et al.*, 1992). mGluR agonists facilitate NMDA receptor mediated responses in hippocampal CA1 neurons (Aniksztejn *et al.*, 1991, 1992; Harvey *et al.*, 1993), rat olfactory cortex (Collins, 1993), neonatal spinal motoneurons (Birse *et al.*, 1993), and in *Xenopus* oocytes expressing rat brain RNA (Kelso *et al.*, 1992). However, the mechanism underlying the facilitation is not uniform across preparations. Facilitation of the NMDA response is mediated by protein kinase C in *Xenopus* oocytes (Kelso *et al.*, 1992), whereas at hippocampal neurons, there is evidence for and against such involvement (Aniksztejn *et al.*, 1991, 1992; Harvey and Collingridge, 1993). In this study, the interaction between NMDA and mGluR agonists was investigated in neocortical neurons along with possible mechanisms underlying the interaction. Receptor desensitization and Ca^{++} sensitivity of the interaction were also studied. This study allows a comparison to be made between two G-protein coupled receptors (5-HT_{2A} and mGluR) identified as facilitating the NMDA response.

7.2 Methods

Wedge preparation, recording, ACSF composition, drug application and analysis of data were as described in sections 2.2., 3.2., 4.2 and 5.2 with the following exception: 2-amino-3-phosphonopropionic acid (D,L-AP3) and 2-amino-4-phosphonobutyric acid (D,L-AP4) were perfused 40 min before addition of agonists. The ACSF contained 0.1 μ M TTX to reduce indirect effects.

7.2.1 Drugs and chemicals

Arachidonic acid, bis-(o-aminophenoxy)-ethane-N,N,N',N', tetraacetic acid (BAPTA), 8-bromo-adenosine cyclic 3',5'-hydrogen phosphate monosodium salt (8-bromo-cAMP), 8-bromo-guanosine 3',5'-hydrogen phosphate monosodium salt (8-bromo-cGMP) were obtained from Sigma. 1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) was from Tocris Neuramin. 2-amino-3-phosphonopropionic acid (D,L-AP3) and 2-amino-4-phosphonobutyric acid (D,L-AP4), bis-(o-aminophenoxy)-ethane-N,N,N',N',tetraacetic acid aminoethoxy (BAPTA-AM) were from Calbiochem. 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was a gift from Ferrosan.

7.3 Results

7.3.1 mGluR receptor agonists selectively facilitate NMDA depolarization

Quisqualate is an agonist at both the mGluR and α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor (Schoepp *et al.*, 1990). Neurons in cortical wedges are depolarized by 10 μ M quisqualate (Harrison and Simmonds, 1985; see Chapter 2) likely reflecting activation of AMPA receptors. However, no depolarization of cortical wedges was observed when 1 μ M quisqualate was perfused alone (data not shown). Nevertheless, 1 μ M quisqualate reversibly facilitated the NMDA depolarization ($192 \pm 13\%$, $n=6$; $p < 0.01$, Fig. 39). Blocking AMPA and kainate receptors with 4 μ M CNQX in the presence of 20 μ M glycine, to protect the NMDA receptor (Thomson, 1990), did not prevent facilitation by 2 μ M quisqualate ($158 \pm 6\%$, $n=4$, $p < 0.05$), although the NMDA depolarization alone was significantly reduced by this treatment ($65 \pm 6\%$, $n=5$, $p < 0.01$). Unlike 10 μ M quisqualate, concentrations of 1S,3R-ACPD (10-200 μ M) did not result in depolarization. Application of 1S,3R-ACPD reversibly enhanced the NMDA depolarization (Fig. 39). Substituting 10 μ M kainic acid for NMDA did not result in facilitation by 50 μ M 1S, 3R-ACPD ($101 \pm 2\%$, $n=5$; Figure 39). As shown in Fig. 40, facilitation of the NMDA response by 1S,3R-ACPD varied in a concentration dependent manner with an EC_{50} of 16 μ M.

In contrast to the facilitation induced by activation of 5-HT_{2A}, muscarinic and α_1 -adrenoceptors (Chapter 2) a second exposure to 1S,3R-ACPD 40 min after the first did not reveal homologous desensitization (Fig. 40). Absence of desensitization allowed a close examination of the facilitation produced by 1S,3R-ACPD. Concentration-response curves for NMDA were obtained with and without 1S,3R-ACPD, employing the same wedge throughout. To allow ready comparison between wedges, the data from each wedge was normalized with the largest response (50 μ M 1S,3R-ACPD plus 50 μ M NMDA) equal to 100%. When examined in this manner, apparently 1S,3R-ACPD shifted the NMDA concentration response curve to the left. (Fig. 41). Dose-ratios for 25 and 50 μ M 1S,3R ACPD were 0.83 and 0.63 respectively and were linear for the concentration range employed (0 to 50 μ M ACPD; F statistic = 594; Fig. 41 insert). Changes in the maximum response could not be ascertained due to loss of the NMDA response following perfusion with elevated concentrations of NMDA (Harrison and Simmonds, 1985).

7.3.2 Antagonism of the 1S,3R-ACPD facilitation

D,L-AP3 and D,L-AP4 antagonize metabotropic glutamate receptor mediated phosphoinositide hydrolysis (Schoepp *et al.*, 1990). Bath application of D,L-AP3 (0.1 to 50 μ M) and D,L-AP4 (1 to 100 μ M) did not alter the NMDA

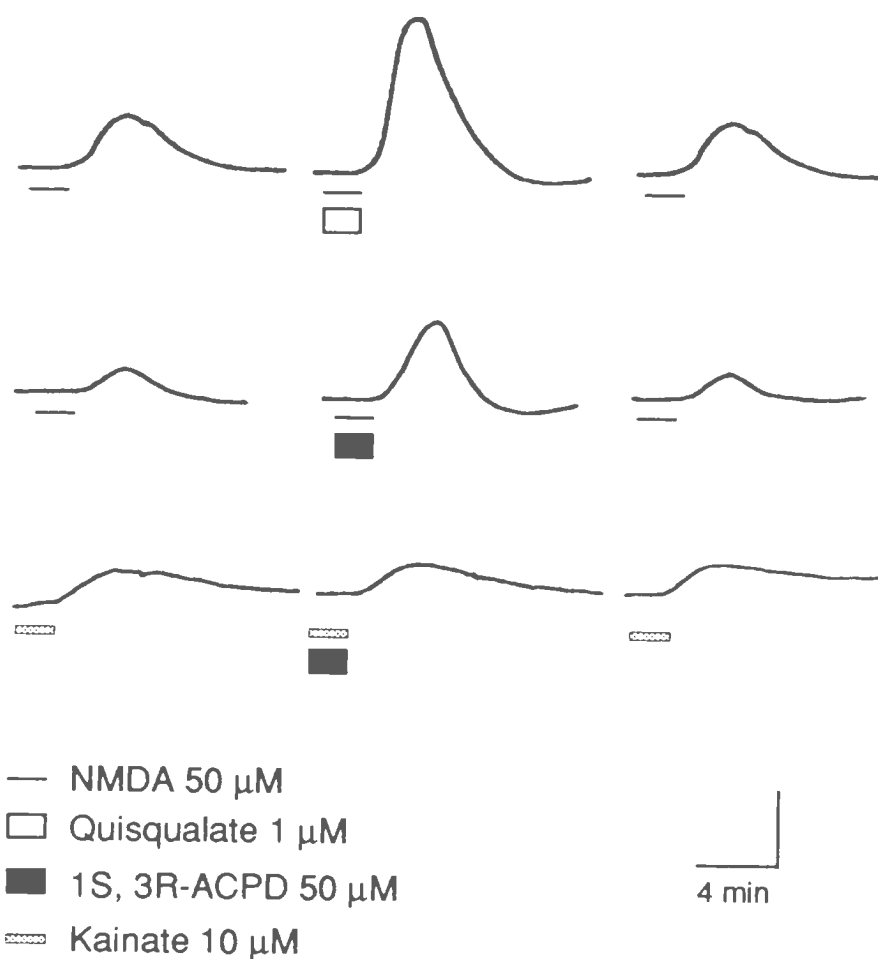


Figure 39. mGluR agonists facilitate the NMDA, but not the kainate induced depolarization of cortical neurons. NMDA (upper two panels) or kainate (lower panel) were applied every 20 min. Quisqualate and 1S,3R-ACPD facilitate NMDA depolarization (upper panels) whereas the kainate depolarization is not facilitated by 1S,3R-ACPD (lower panel). Data from 3 wedges. Voltage calibration is 2 mV for upper panels, 1 mV for lower panel.

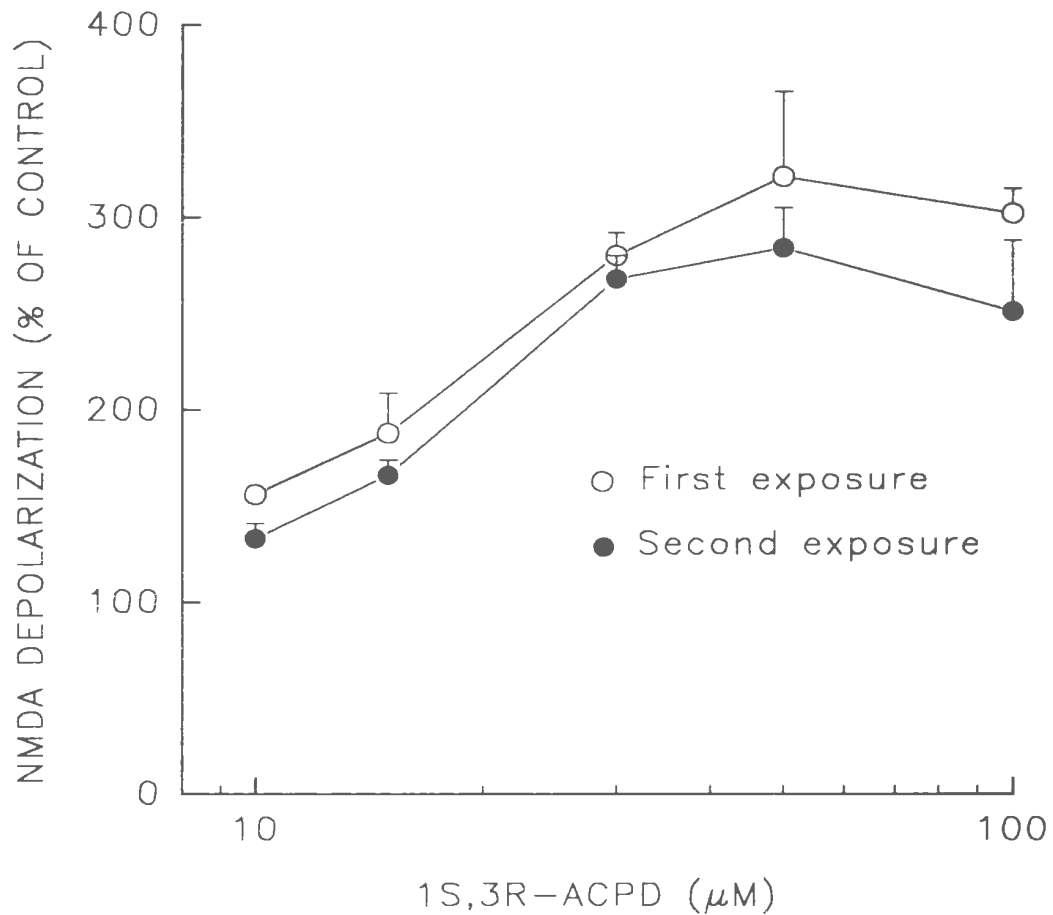


Figure 40. Concentration-dependent facilitation of the NMDA depolarization induced by 1S,3R-ACPD and lack of desensitization. Each wedge was exposed to the same concentration of 1S,3R-ACPD plus 50 μ M NMDA two times separated by 20 min. Note there is only a small reduction (not significant) in the facilitation on second exposure. 5 to 8 wedges used for each concentration.

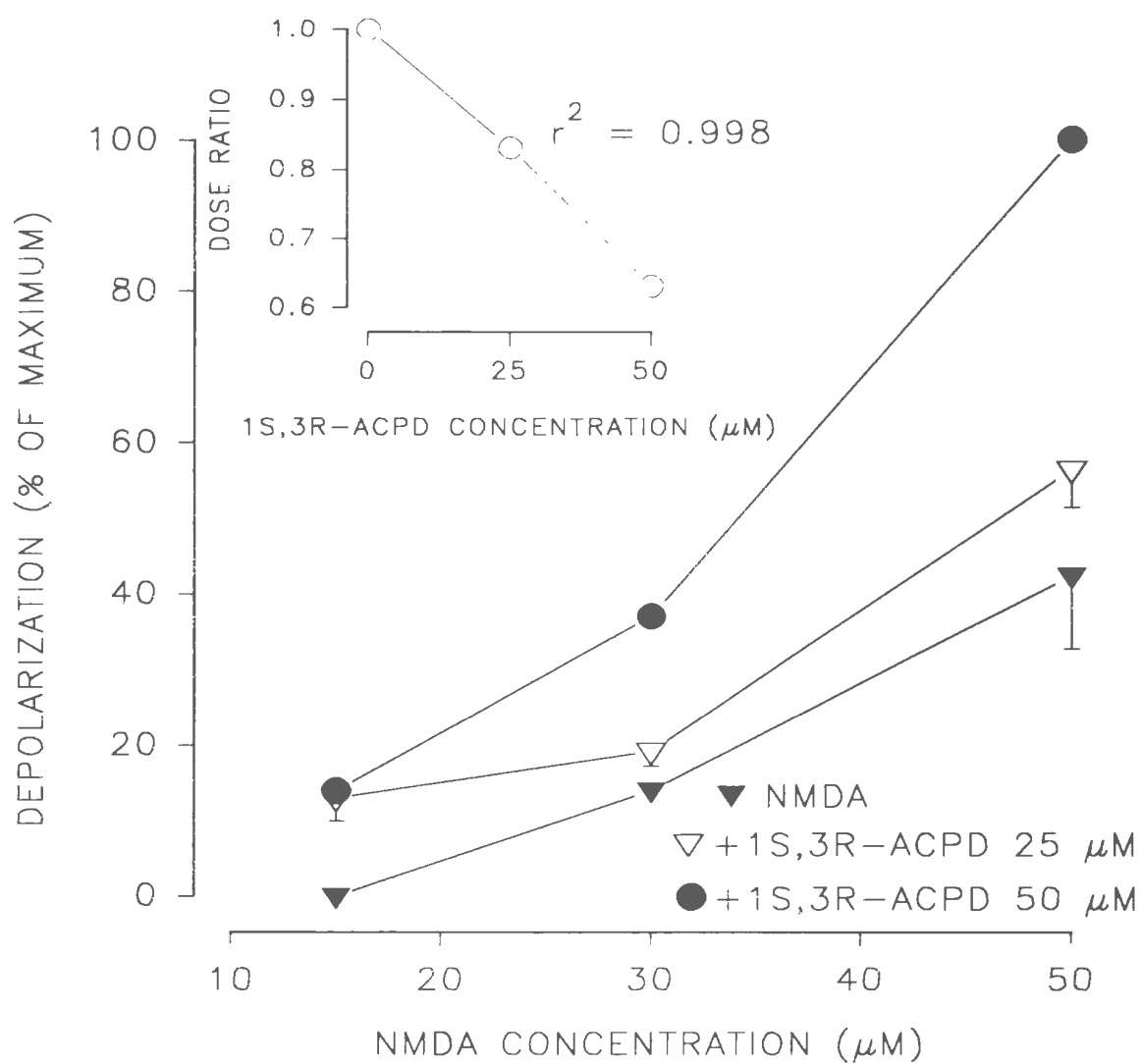


Figure 41. Concentration-dependent facilitation of the NMDA depolarization induced by 1S,3R-ACPD. Concentration response curves are shown for NMDA and 1S,3R-ACPD. Responses are expressed as a % of maximum. Values are the mean \pm SEM from 3 separate experiments.

depolarization ($98 \pm 2\%$, $n=10$). However, both agents reduced 1S,3R-ACPD facilitation of the NMDA response in a concentration-dependent manner (Figure 42A). The IC_{50} values for D,L-AP3 and D,L-AP4 were 0.24 and 4.4 μ M respectively. Varying the concentration of 1S,3R-ACPD in the presence of D,L-AP3 (Fig. 42B) and D,L-AP4 (not shown) revealed that the antagonism was not competitive.

7.3.3 Facilitation by 1S,3R-ACPD does not exhibit desensitization

The lack of homologous desensitization observed with 1S,3R-ACPD prompted us to investigate other agents which modify desensitization of 5-HT_{2A} receptors (Chapter 3 and 4). Prolonged perfusion with 3 μ M phorbol diacetate did not significantly depress the 1S, 3R-ACPD facilitation (Table V) compared to the same treatment in the presence of staurosporine. Similarly, perfusing 10 nM concanavalin A or 1 μ M chlorpromazine failed to alter the facilitation induced by 30 μ M 1S,3R-ACPD (Table V).

In addition to homologous desensitization, the facilitation induced by carbachol and phenylephrine is subject to heterologous desensitization (Chapters 3 and 4). This form of desensitization results from substrate depletion following exposure to a high (100 μ M) concentration of 5-HT (Chapter 4). As shown in Fig. 43, the 1S,3R-ACPD induced facilitation was also eliminated by exposure to 100 μ M 5-HT. On the other hand, perfusing wedges with 10 mM myo-inositol, which

Figure 42. Antagonism of the 1S,3R-ACPD facilitation. **A.** D,L-AP3 and D,L-AP4 reduce the 1S,3R-ACPD facilitation in a concentration dependent manner. NMDA depolarization in the presence of antagonists was used as control. 4 to 6 wedges were used at each antagonist concentration. ***, $p < 0.001$. **B.** Dose response curves are shown for 1S,3R-ACPD, D,L-AP3 1 μ M and 10 μ M. Both concentrations produced non competitive antagonism. Values are from 5 separate experiments.

Figure 42. Antagonism of the 12,13-ACPD facilitation. A. DJ-AP3 and DJ-AP4 reduce the 12,13-ACPD facilitation in a concentration dependent manner. BMDA depolarization in the presence of antagonists was used as control. 4 to 6 wedges were used at each antagonist concentration. $^{***}p < 0.001$. B. Dose response curves are shown for 12,13-ACPD, DJ-AP3 1 μ M and 10 μ M. Both concentrations produced non competitive antagonism. Values are from 3 separate experiments.

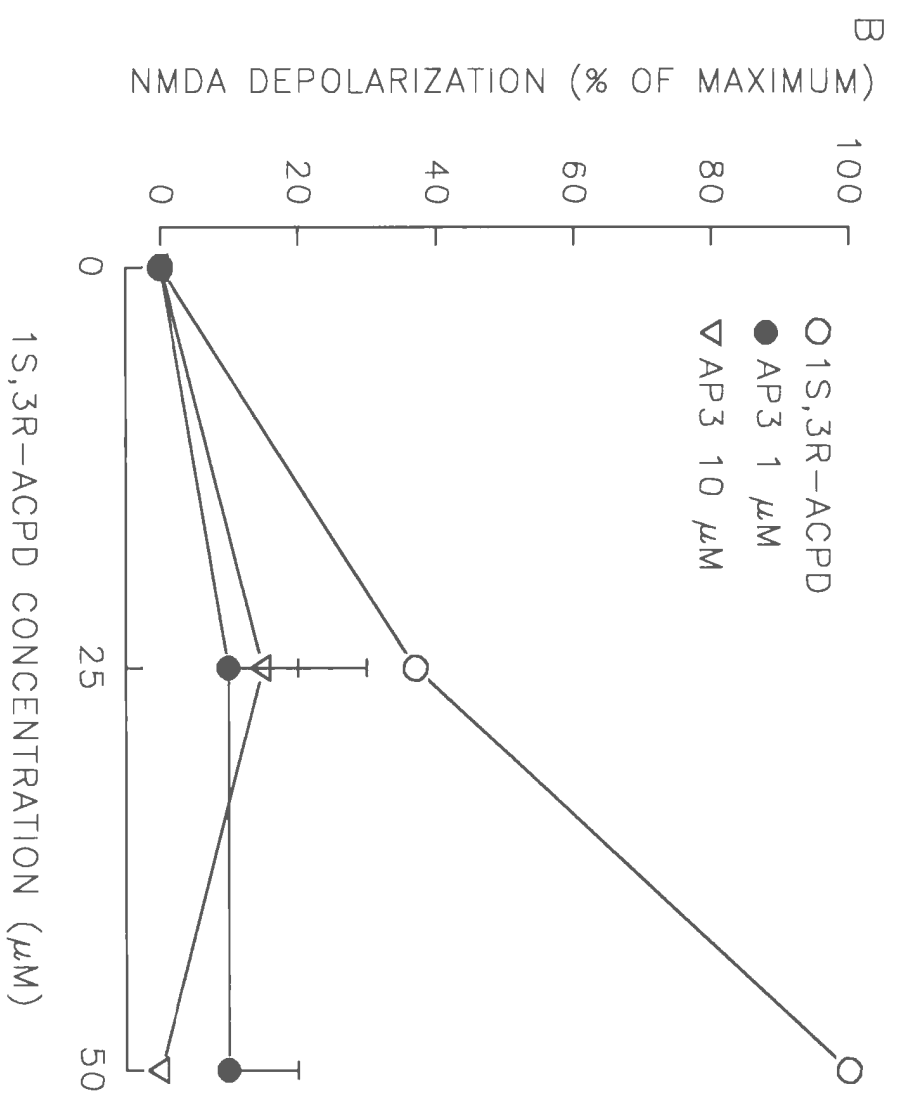
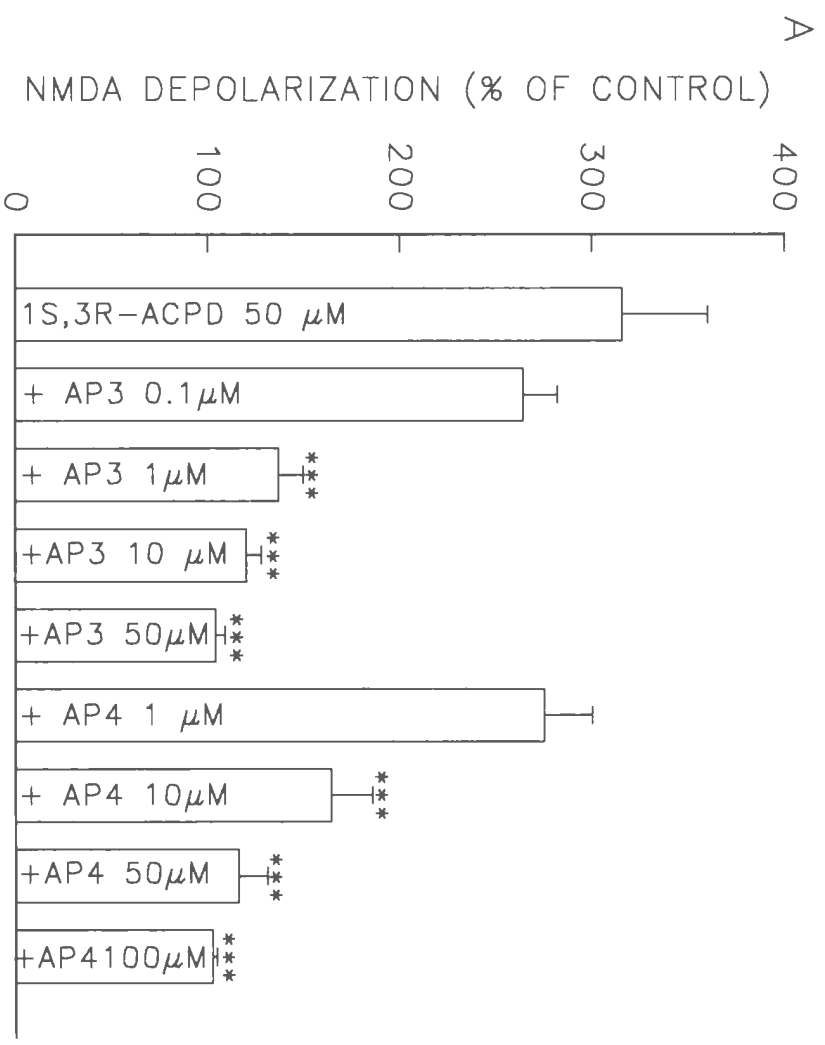


Table V. Effects of phorbol diacetate, staurosporine, concanavalin A and chlorpromazine on 1S,3R-ACPD facilitation.

Drugs	NMDA depolarization (% of control)
1S,3R-ACPD (30 μ M)	266 \pm 24% (n = 8)
+ Phorbol diacetate (3 μ M)	228 \pm 21% (n = 5)
+ Staurosporine (10 nM)	271 \pm 19% (n = 5)
+ Concanavalin A (100 nM)	260 \pm 18% (n = 5)
+ Chlorpromazine (1 μ M)	251 \pm 22% (n = 4)

Statistical comparisons were performed by one-way analysis of variance followed by Bonferroni test. All treatments were compared with 1S,3R-ACPD (control) except phorbol diacetate which was compared with staurosporine. Neither group was significantly different.

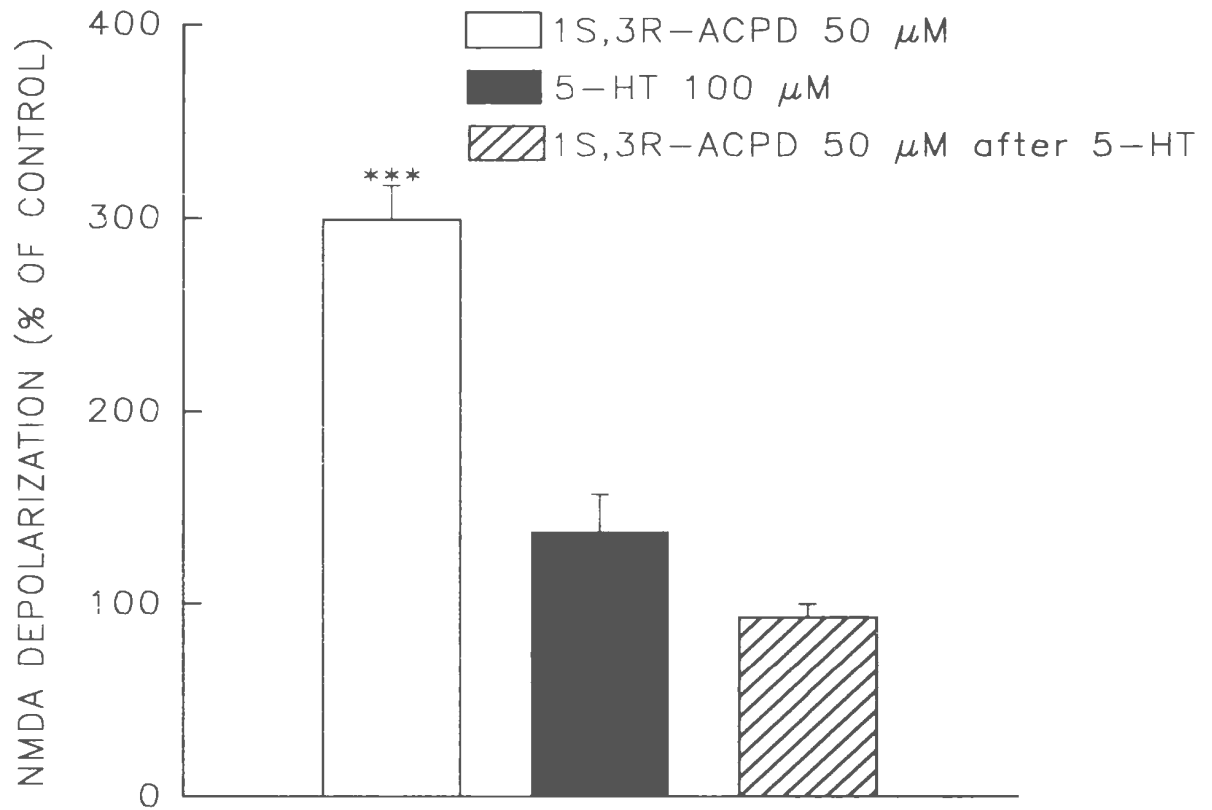


Figure 43. 1S,3R-ACPD (50 μ M) facilitation before and after 5-HT (100 μ M). 1S,3R-ACPD facilitation was eliminated after 5-HT application. 4 wedges were used in each treatment. ***, $p < 0.001$, NMDA vs NMDA +1S,3R-ACPD.

enhances availability of substrate for agonist induced phosphoinositide hydrolysis (Fain and Berridge, 1979; Pontzer and Crews, 1990; Chapter 4), resulted in a significant potentiation of the 1S,3R-ACPD induced facilitation ($379 \pm 12\%$, $n=4$, $p < 0.05$).

In addition to coupling to phospholipase C, mGluRs also couple to phospholipase A_2 and adenylate cyclase (Aramori and Nakanishi, 1992). Moreover, arachidonic acid and cyclic AMP have been implicated in the enhancement of NMDA responses (Miller *et al.*, 1992; Cerne *et al.*, 1993). However, application of arachidonic acid or 8-bromo-cAMP or 8-bromo-cGMP (permeable analogues of cAMP or cGMP) failed to facilitate the NMDA depolarization (Fig. 44).

7.3.4 Ca^{++} dependence of the 1S,3R-ACPD mediated facilitation

A rise in intracellular Ca^{++} is thought to underlie facilitation of the NMDA response induced by 5-HT in cortical neurons (Chapter 5) and by acetylcholine in hippocampal neurons (Markram and Segal, 1992). To investigate the importance of external Ca^{++} for the facilitation, 1S,3R-ACPD was applied following (40-60 min) perfusion with Ca^{++} free ACSF. This treatment did not reduce the 1S,3R-ACPD or carbachol induced facilitation of the NMDA response (Fig. 45A). On the other hand, the facilitation induced by 5-HT and phenylephrine was abolished

during perfusion with Ca^{++} free ACSF (Fig. 45). Addition of 100 μM BAPTA to the nominally Ca^{++} free ACSF gradually eliminated the 1S,3R-ACPD mediated facilitation ($75 \pm 7\%$, $n=4$, $p < 0.05$; Fig. 46, upper panel) over the course of one hour. Ca^{++} influx through voltage operated Ca^{++} channels is necessary for the 5-HT facilitation (Chapter 5). Although 10 μM nifedipine significantly reduced the NMDA response ($73 \pm 15\%$, $n=6$; $p < 0.05$), the facilitation induced by 30 μM 1S,3R-ACPD was not eliminated ($221 \pm 8\%$, $n=4$; $p < 0.05$; Fig. 46, middle panel). Finally, the importance of intracellular Ca^{++} to the facilitation was examined by perfusing cell permeant BAPTA-AM. BAPTA is formed by deesterification of BAPTA-AM inside the cell (Niesen *et al.*, 1991). BAPTA-AM (50 μM) eliminated the 1S,3R-ACPD induced facilitation (Fig. 46, lower panel). BAPTA-AM did not alter the NMDA depolarization ($97 \pm 2.5\%$, $n=5$).

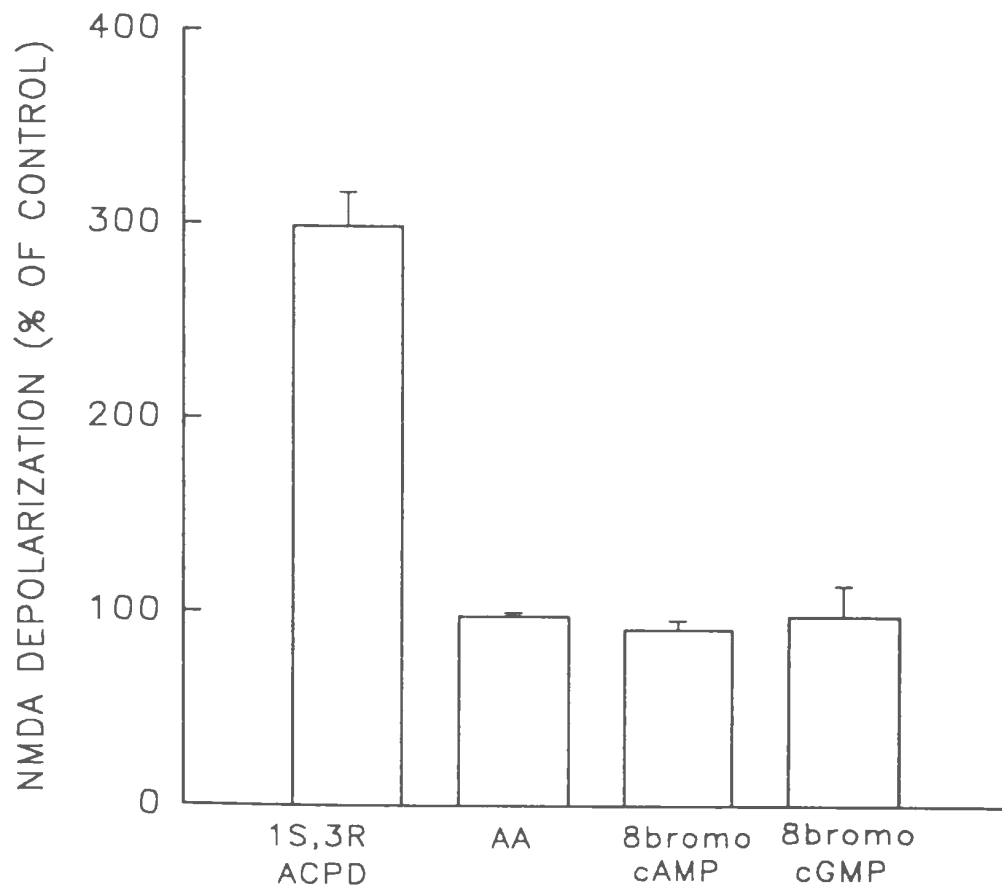


Figure 44. Arachidonic acid (AA;10 μ M), 8-bromo-cAMP (20 μ M), or 8-bromo-cGMP (20 μ M) do not mimic the facilitation induced by 1S,3R-ACPD. Each agent was co-applied with 50 μ M NMDA for 2 min. 4 to 5 wedges were used for each treatment.

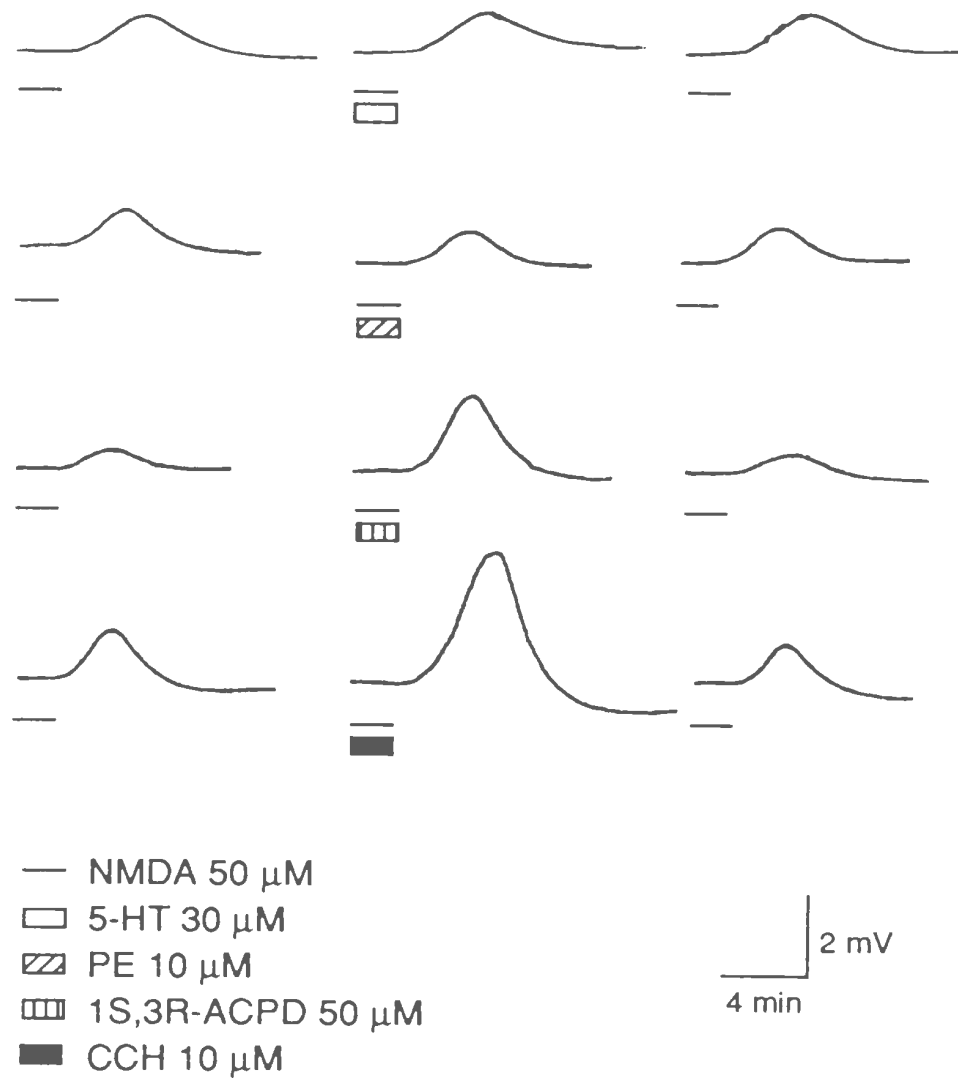
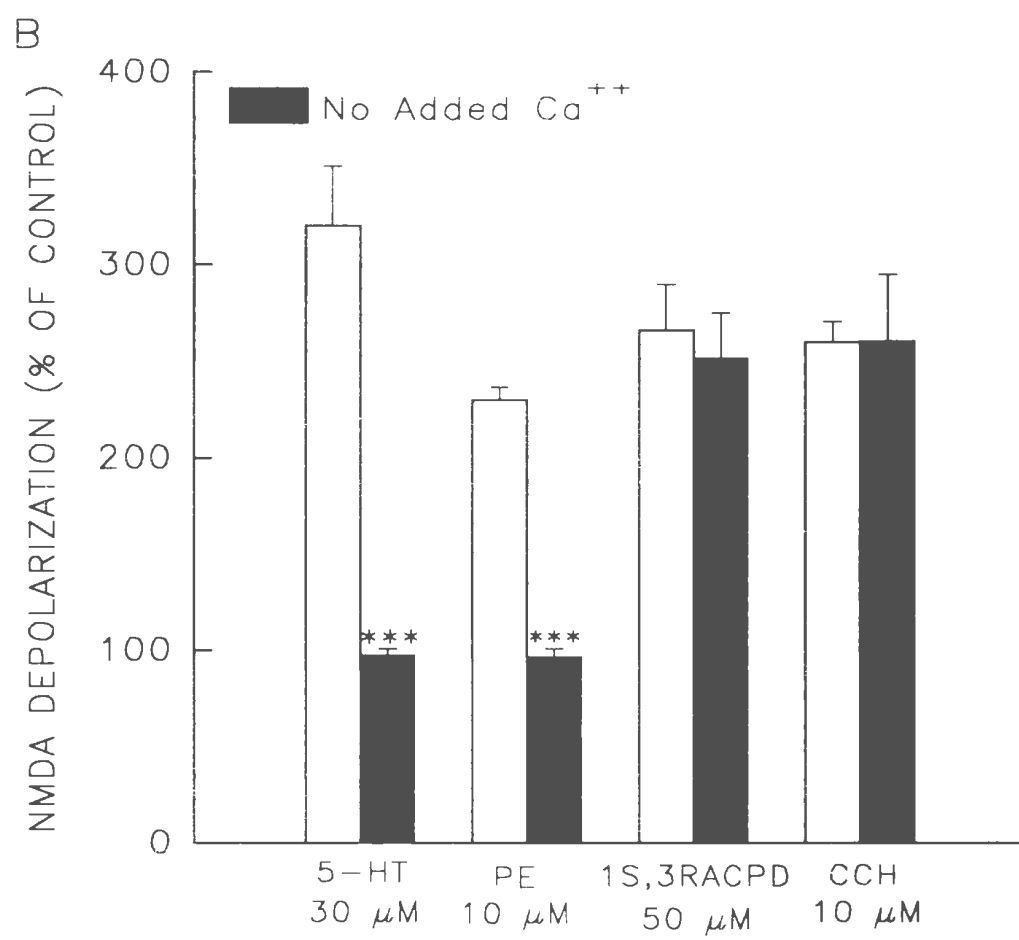


Figure 45. Effect of Ca^{2+} free ACSF (no added Ca^{2+}) on 5-HT, phenylephrine (PE), 1S,3R-ACPD and carbachol (CCH) facilitation. **A.** Only 1S,3R-ACPD and CCH facilitation persisted in the absence of extracellular Ca^{2+} . **B.** Average data for 5-HT, PE, 1S, 3R-ACPD and CCH. 4 to 6 wedges were used in each case. ***, $p < 0.001$.



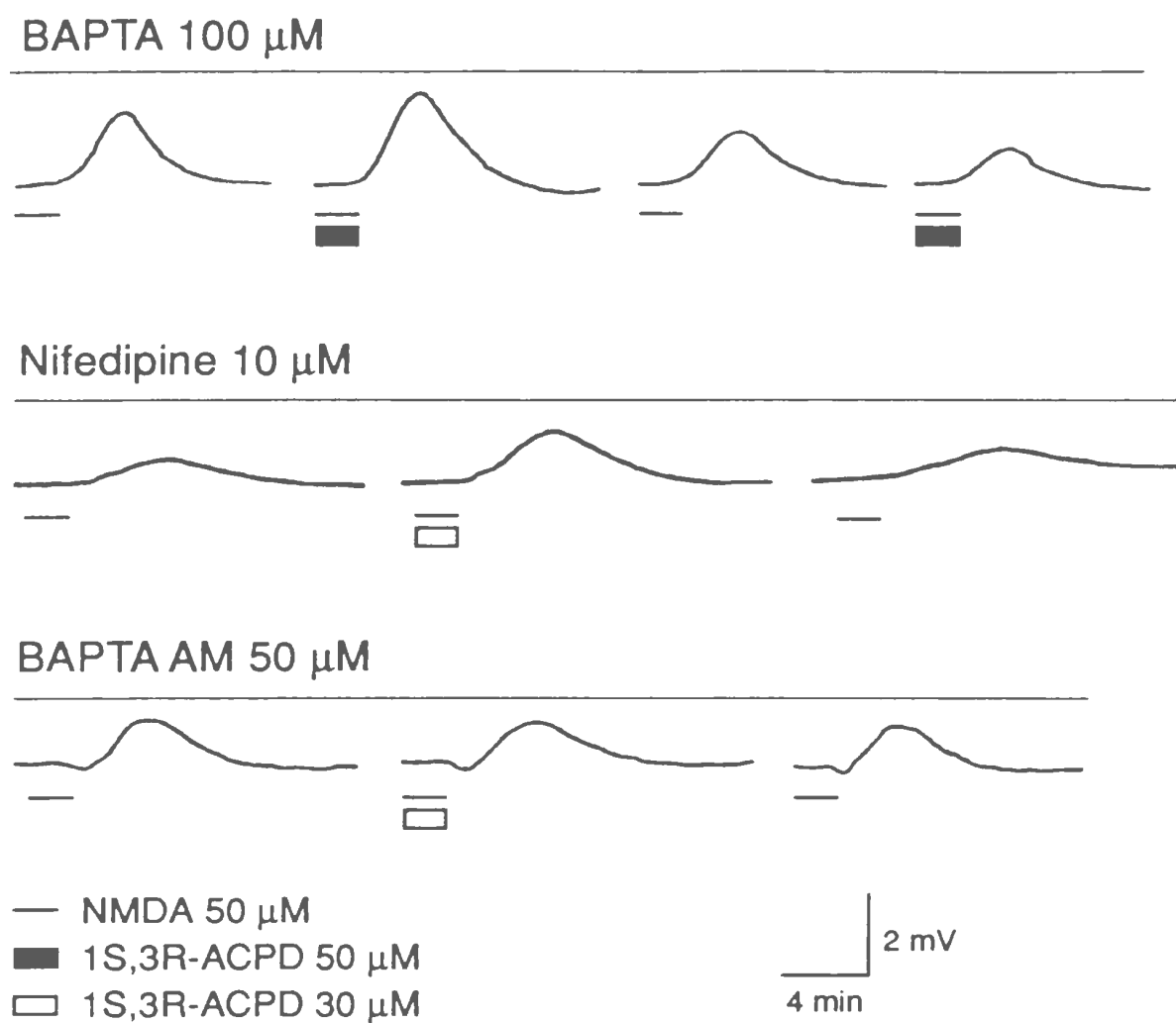


Figure 46. Effect of BAPTA, nifedipine and BAPTA-AM on 1S,3R-ACPD facilitation. 1S,3R-ACPD facilitation was reduced on first exposure and eliminated on second exposure following addition of BAPTA to Ca^{++} free ACSF (upper panel). Note the NMDA depolarization was also dramatically reduced. Nifedipine failed to eliminate 1S,3R-ACPD facilitation (middle panel). Unlike BAPTA, BAPTA-AM eliminated the 1S,3R-ACPD facilitation completely on first exposure (lower panel). 4 to 5 wedges were used in each condition.

7.4 Discussion

7.4.1 Characterization of the 1S,3R-ACPD facilitation

The present experiments demonstrate that application of the mGluR agonists quisqualate and 1S,3R ACPD selectively facilitate the depolarization of cortical neurons induced by NMDA. The facilitation was manifest as a 1S,3R-ACPD concentration dependent shift of the NMDA concentration-response curve to the left. Moreover, the facilitation was observed at concentrations of quisqualate and 1S,3R-ACPD at which neither agonist produced an overt depolarization on its own. Although not examined in detail (see section 7.3.1), the order of potency appears to be quisqualate > 1S,3R-ACPD. 1S,3R-ACPD is a selective agonist at mGluR (Palmer *et al.*, 1989; Irving *et al.*, 1990), whereas quisqualate is an agonist at both AMPA and metabotropic receptors (Murphy and Miller, 1989). However, in the presence of an AMPA/kainate antagonist, quisqualate remains effective in facilitating the NMDA response. The smaller than expected facilitation observed with CNQX doubtless reflects loss of a small contribution from the AMPA receptor activation. Quisqualate enhances formation of inositol polyphosphates along with Ca^{++} entry and mobilization, in part, through its action on AMPA receptors (Baird *et al.*, 1991; Murphy and Miller, 1988).

The facilitatory response induced by 1S,3R-ACPD was antagonized

noncompetitively by both D,L-AP3 and D,L-AP4. Neither antagonist altered the depolarization induced by NMDA in the absence of 1S,3R-ACPD indicating that, under the conditions of the present experiments, there was insufficient transmitter release in the wedges to activate mGluR and facilitate the NMDA response. This is not the case for other receptors in cortical wedges, e.g. both scopolamine and prazosin significantly reduce the amplitude of the NMDA depolarization (Chapter 2). Surprisingly, the IC_{50} values for D,L-AP3 and D,L-AP4 antagonism of the 1S,3R-ACPD facilitation are orders of magnitude lower than previously reported for mGluR mediated responses (Schoepp *et al.*, 1990; Schoepp and Conn, 1993). Whether this reflects the nature of the response examined, i. e. phosphoinositide hydrolysis versus facilitation of the NMDA depolarization, is not clear. D,L-AP3 and D,L-AP4 are reportedly weak partial agonists (Schoepp *et al.*, 1990; Schoepp and Conn, 1993). However, within the concentration range of D,L-AP3 and D,L-AP4 examined, no partial agonism was observed with respect to facilitating the NMDA response.

In keeping with observations on hippocampal neurons (Aniksztejn *et al.*, 1991, 1992; Harvey and Collingridge, 1993), spinal neurons (Bleakman *et al.*, 1992) and *Xenopus* oocytes (Kelso *et al.*, 1992), the above observations are compatible with quisqualate and 1S,3R-ACPD acting at mGluR to facilitate the NMDA depolarization. Presumably the mGluR mediating the facilitation acts

directly on projection neurons passing through the grease seal (see Chapter 2) since the facilitation was present in nominally Ca^{++} free ACSF containing tetrodotoxin. mGluR couple through G-proteins to ion channels and a variety of second messenger systems including phospholipase C and D, adenylate cyclase (both positive and negative coupling) and arachidonic acid (Schoepp and Conn, 1993). Activation of phospholipase C or adenylate cyclase as well as application of arachidonic acid has been shown to result in facilitation of the NMDA response in different brain regions (Markram and Segal, 1992; Chapter 3; Cerne *et al.*, 1993; Miller *et al.*, 1992). However, our observations demonstrate that in cortical wedges 8-bromo-cAMP, 8-bromo-cGMP and arachidonic acid are unable to substitute for 1S,3R-ACPD in evoking a facilitation. These findings are in agreement with data from CA1 hippocampal neurons (Harvey and Collingridge, 1993). Involvement of phospholipase D is also unlikely since L-AP3 is a full agonist at mGluR that couples to phospholipase D (Boss and Conn, 1992).

7.4.2 Potentiation of 1S,3R-ACPD facilitation by myo-inositol

On the positive side, myo-inositol potentiates the 1S,3R-ACPD facilitation, whereas prior exposure to 5-HT eliminates it. Both results are consistent with the hypothesis that activation of phospholipase C is critical for the facilitation (Chapter 2). Myo-inositol is a precursor of phosphoinositide 4,5-biphosphate, the substrate

for phospholipase C (Berridge, 1993). Brain slices incubated in ACSF exhibit lower inositol levels than fresh brain tissue unless 10 mM myo-inositol is present (Sherman *et al.*, 1986). Enhancing substrate availability by perfusing hippocampal slices with myo-inositol results in greater inositol phosphate formation following carbachol stimulated phosphoinositide hydrolysis (Pontzer and Crews, 1990). As with the 1S,3R-ACPD facilitation, the NMDA facilitation mediated by receptors employing phospholipase C for signal transduction at cortical neurons is potentiated by myo-inositol (see Chapter 4). On the other hand, stimulating phospholipase C with high concentrations of an agonist such as 5-HT, presumably reducing substrate availability in the process (Fain and Berridge, 1979), results in heterologous desensitization of the carbachol and phenylephrine facilitation at cortical neurons. This desensitization is prevented by addition of 10 mM myo-inositol to the perfusion medium (see Chapters 3 and 4). Thus, the potentiation by myo-inositol and the heterologous desensitization induced by 5-HT is entirely consistent with the 1S,3R-ACPD facilitation resulting as a consequence of stimulating phospholipase C. Moreover, the EC_{50} value for the 1S,3R-ACPD facilitation (16 μ M) is in good agreement with the EC_{50} values reported for 1S,3R-ACPD stimulated phosphoinositide hydrolysis in primary cerebrocortical cultures (15 μ M; Birrell and Marcoux, 1993) and neonatal rat cerebral cortex slices (16 μ M; Challiss *et al.*, 1994). Finally, D,L-AP3 and D,L-AP4 noncompetitively inhibit

mGluR induced phosphoinositide hydrolysis in cortical tissue (Schoepp *et al.*, 1990, and 1990a) and it seems reasonable to conclude that these antagonists behave in a similar manner in the present study.

7.4.3 Involvement of PLC signal transduction

Of the mGluR so far identified, only the mGluR1 and the mGluR5 subtypes couple to phospholipase C (Abe *et al.*, 1992; Aramori and Nakanishi, 1992; Schoepp and Conn, 1993; Tanabe *et al.*, 1992). The mGluR5 subtype is abundantly present in the cerebral cortex (Abe *et al.*, 1992; Minakami *et al.*, 1993), whereas the mGluR1 subtype is found at a much lower level. Agonist potency (quisqualate > 1S,3R-ACPD) for both receptors is similar to observations in the present experiments (Abe *et al.*, 1992; Aramori and Nakanishi, 1992). Chinese hamster ovary cells transfected with mGluR1 or mGluR5 are insensitive to L-AP3, suggesting that L-AP3 acts at a site separate from the mGluR or that another mGluR, which remains to be identified, couples positively to phospholipase C. Assuming the first case is correct, the present observations cannot distinguish between the mGluR1 and mGluR5 with respect to the subtype mediating the facilitation in cortical wedges.

Phosphoinositide specific phospholipase C plays a crucial role in initiating the surface mediated signal transduction by generating second messenger

molecules, diacylglycerol, and inositol 1,4,5-triphosphate (Wojcikiewicz *et al.*, 1993). Catalytic activation of phospholipase C is dependent on Ca^{++} concentration and results accumulated during the last few years clearly indicate that there are a number of distinct variants of phospholipase C which have been purified, cloned and sequenced (Rhee *et al.*, 1991). Carbachol, norepinephrine, and 5-HT stimulated PI hydrolysis in cortical slices exhibit different requirements for external Ca^{++} (Kendall and Nahorski, 1984). Furthermore, 1S,3R-ACPD has been shown to stimulate phosphoinositide hydrolysis that is not dependent on extracellular Ca^{++} (Birrell and Marcoux, 1993). The distinct Ca^{++} sensitivity associated with the different receptors with respect to the NMDA facilitation presumably indicates different phospholipase C subtype(s) are involved in signal transduction rather than that the Ca^{++} requirements for the facilitation differ in a significant manner between receptors.

7.4.4 Role of external and internal Ca^{++}

The effectiveness of BAPTA-AM in eliminating the 1S,3R-ACPD facilitation clearly suggests that an intracellular Ca^{++} -dependent process is necessary for the facilitation. 1S,3R-ACPD raises intracellular Ca^{++} by generating inositol 1,4,5 triphosphate which then acts to release Ca^{++} from the smooth endoplasmic reticulum (Irving *et al.*, 1990; Berridge, 1993). It is proposed that the inositol 1,4,5

triphosphate evoked release of Ca^{++} is responsible for the facilitation and that BAPTA-AM acts to buffer this rise in Ca^{++} , thus preventing the facilitation from taking place. This is consistent with the muscarinic cholinergic mediated facilitation in the hippocampus which is blocked by BAPTA applied through the recording electrode (Markram and Segal, 1992). No evidence was obtained in the present experiment for involvement of the other arm of PI pathway, i.e., diacylglycerol stimulated protein kinase C. Phorbol diacetate did not occlude the 1S,3R-ACPD facilitation and staurosporine did not block it. This is in keeping with the 1S,3R-ACPD induced facilitation of the NMDA response in the cerebellum (Kinney and Slater, 1993), but differs from reports on the 1S,3R-ACPD facilitation in *Xenopus* oocytes and hippocampal neurons (Kelso *et al.*, 1992; Aniksztejn *et al.*, 1991, 1992).

In contrast to the requirement for a rise in intracellular Ca^{++} , the 1S,3R-ACPD facilitation does not require external Ca^{++} . This is consistent with reports that 1S,3R-ACPD induced phosphoinositide hydrolysis is not dependent on extracellular Ca^{++} (Birrell and Marcoux, 1993; Challiss *et al.*, 1994). Insensitivity to external Ca^{++} also suggests that Ca^{++} entry through the NMDA receptors channel is not important for the facilitation.

1S,3R-ACPD acting at mGluR blocks voltage dependent Ca^{++} channels (L-type) in cortical pyramidal neurons thereby occluding the blockade by nifedipine

(Sayer *et al.*, 1992). In keeping with these observations, nifedipine did not reduce the 1S,3R-ACPD facilitation. In this regard, the facilitation mediated by 5-HT_{2A} receptors is quite distinct in that it is blocked in a concentration-dependent manner by nifedipine (Chapter 5). Clearly, further investigation is required to resolve these differences.

In this study, changes in the NMDA response were not observed initially as demonstrated (see Chapter 5) in the presence of Ca⁺⁺ free medium containing BAPTA-AM. However, the NMDA response was reduced following multiple exposures to the agonist. A plausible explanation for this discrepancy is that the membrane depolarization and loss of the cholinergic and noradrenergic components induced by the Ca⁺⁺ free medium are no longer operative (Chapter 5). Therefore, the addition of BAPTA-AM in the medium would not change the NMDA response initially. However, the subsequent reduction is likely due to the continuous perfusion of BAPTA-AM which could further reduce the concentration of internal Ca⁺⁺ (see Chapter 5). This alteration in internal Ca⁺⁺ might account for the subsequent reduction of the NMDA depolarization in Ca⁺⁺ free medium with BAPTA-AM.

7.4.5 1S, 3R-ACPD facilitation and homologous desensitization

An interesting difference between the present findings and previous results

with 5-HT_{2A}, α_1 -adrenoceptor, and muscarinic cholinergic agonists is the apparent lack of homologous desensitization of mGluR (Chapter 3). Moreover, activating protein kinase C totally eliminates the 5-HT induced facilitation by an action on the 5-HT_{2A} receptor (see Chapter 3). Despite reports of protein kinase C regulating mGluR responses (Manzoni *et al.*, 1990, 1991), no evidence for potentiation or suppression of the facilitation by inhibiting or stimulating protein kinase C was observed. Thus, in cortical neurons the feedback regulation of metabotropic receptors by protein kinase C is absent. Consistent with this view, the mGluR1 and mGluR5 sequences reveal only low probability consensus sequences for protein kinase C phosphorylation (Kemp and Pearson, 1990).

In conclusion, the present findings suggest that the 1S,3R-ACPD facilitation in the cortex is dependent on phospholipase C signal transduction and a rise in intracellular Ca²⁺. In this regard, the mGluR induced facilitation closely resembles that induced by other G-protein coupled receptors which couple positively to phospholipase C.

CHAPTER 8

GENERAL DISCUSSION AND FUTURE DIRECTIONS

The above investigations have furnished new insights into the functional interaction between NMDA receptors and 5-HT_{2A} receptors and other G-protein coupled receptors positively coupled to PLC in rat neocortex. The major findings of this research are summarized as follows:

1. Activation of 5-HT_{2A} receptors selectively facilitates the NMDA induced depolarization of cortical projection (pyramidal) neurons and this is likely to be a direct effect since, at least in layer V, 5-HT_{2A} receptors are located on pyramidal neurons.
2. Among the consequences of activating 5-HT_{2A} receptors is a rise in intracellular Ca⁺⁺. This event likely accounts for the facilitation. Moreover, the results suggest that protein kinase C, calmodulin, arachidonic acid, cyclic AMP and cyclic GMP are not responsible for the facilitation.
3. 5-HT_{2A} receptor stimulated signal transduction is selectively altered during

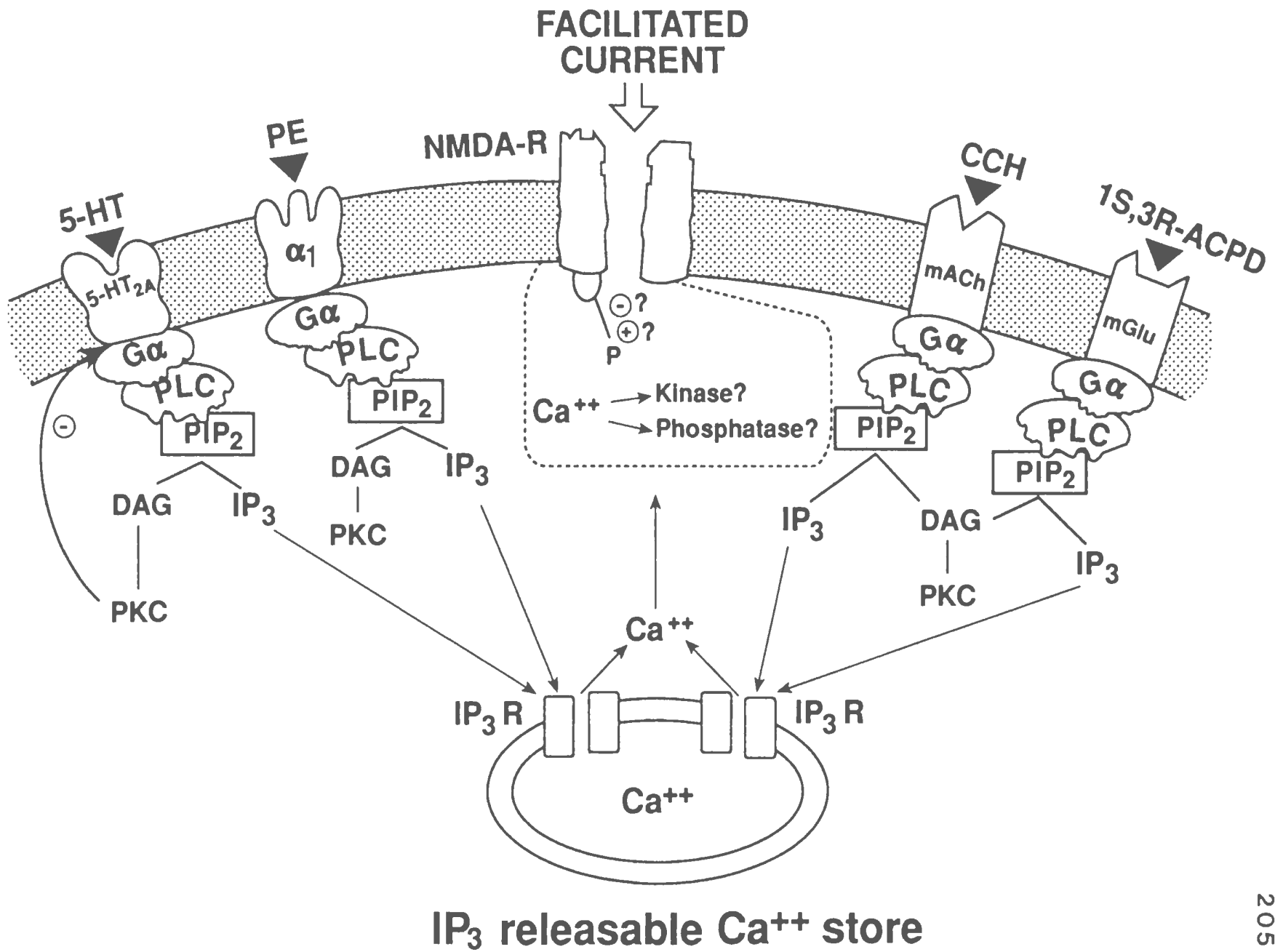
aging; activation of protein kinase C is thought to underlie this alteration. This is consistent with evidence for negative feedback regulation of 5-HT_{2A} receptors by protein kinase C. However, feed-forward regulation of protein kinase C by another transmitter/modulator cannot be excluded.

4. Like 5-HT_{2A} receptors, activation of muscarinic cholinceptors, α_1 -adrenoceptors and mGluR facilitate NMDA responses in cortical neurons. All of these receptors appear to induce the facilitation through the IP₃-Ca⁺⁺ pathway. Unlike 5-HT_{2A} receptors, these other receptors are insensitive to activation of protein kinase C, at least with respect to the NMDA facilitation. Although a rise in Ca⁺⁺ appears to underlie the facilitation induced by these receptors, the importance of external Ca⁺⁺ and entry through voltage dependent Ca⁺⁺ channels varies from receptor to receptor. The action of these receptors is summarized in Fig. 47.

How a rise in intracellular Ca⁺⁺ is followed by NMDA facilitation is not understood. A number of processes might account for facilitation of the NMDA response. The rise in intracellular Ca⁺⁺ probably activates various protein kinases and phosphatases which could regulate the NMDA receptor (Wang *et al.*, 1994; Wang and Salter, 1994; Lieberman and Mody, 1994).

Figure 47. Schematic diagram of 5-HT_{2A}, α₁-adrenoceptor, mGluR and muscarinic cholinergic stimulated pathways that account for the NMDA facilitation.

Abbreviations: IP₃R, inositol 1,4,5 triphosphate receptor; Gα, α subunit of G-protein; DAG, diacylglycerol; PKC, protein kinase C.



Protein kinase C has been implicated in the enhancement of the NMDA response in hippocampus, trigeminal neurons and *Xenopus* oocytes (Aniksztejn *et al.*, 1991, 1992; Chen and Huang, 1992; Kelso *et al.*, 1992; Durand *et al.*, 1992). Protein kinase C in some cases phosphorylates NMDA receptors thereby enhancing current flow through the channel (Chen and Huang, 1992; Kelso *et al.*, 1992; Durand *et al.*, 1992). However, there is no evidence for such a mechanism in the case of neocortical projection neurons (see Chapters 3, 4, 6 and 7). Protein kinase C does influence 5-HT_{2A} receptors, since protein kinase C inhibitors potentiate the NMDA facilitation (Chapters 3 and 6). This suggests that 5-HT_{2A} receptors undergo phosphorylation that is blocked by these inhibitors as has been observed at 5-HT_{2A} receptors in other parts of the central nervous system (Aghajanian, 1990; Marek and Aghajanian, 1994). Similarly, protein kinase C inhibition potentiates cholinergic facilitation at hippocampal neurons (Markram and Segal, 1992). Calmodulin does not appear to be important for the facilitation in neocortex, whereas it may be involved with long term desensitization of 5-HT_{2A} receptors (see Chapter 3) and the biphasic nature of the facilitation observed with thapsigargin, cyclopiazonic acid and A23187 (Chapter 5)

Recently, tyrosine kinase and a number of phosphatases have been shown to regulate NMDA receptor function (Wang and Salter, 1994; Wang *et al.*, 1994; Lieberman and Mody, 1994). Involvement of tyrosine kinase in the 5-HT_{2A}

facilitation is unlikely, since genistein, an inhibitor of this enzyme, is ineffective in enhancing or blocking the 5-HT facilitation (Rahman and Neuman, unpublished observations). Inhibition of protein phosphatases 1 and 2A with calyculin A enhances the peak NMDA current, but does not shift the NMDA concentration-response curve (Wang *et al.*, 1994). On the other hand, 5-HT and 1S,3R-ACPD both shift the NMDA concentration-response curve to the left. Moreover, agonist induced facilitation of the NMDA response, up to 700%, is far greater than the increase in NMDA current induced by tyrosine kinase or phosphatases (Wang and Salter, 1994; Wang *et al.*, 1994). Clearly, further study is required to establish how the NMDA response is enhanced.

Significance of 5-HT_{2A} receptor mediated facilitation

1. Neocortical plasticity

NMDA receptors are the key elements in many aspects of central nervous system plasticity (Malenka and Nicoll, 1993). Endogenous modulators such as acetylcholine, glutamate acting at mGluR, noradrenaline and 5-HT may play an important role in influencing synaptic plasticity dependent on NMDA receptors. Acetylcholine, noradrenaline and 1S,3R-ACPD, for example, facilitate development of long term potentiation (Bear and Singer, 1986; Brocher *et al.*, 1992; Schoepp and Conn, 1993), whereas 5-HT, acting at 5-HT_{2A} receptors, produces long lasting

changes in cortical NMDA responses (Reynolds *et al.*, 1988). Whether 5-HT_{2A} receptor activation can facilitate long term potentiation in cortical neurons has not been examined. However, it would be surprising if this were not the case. Consistent with this notion, it appears that 5-HT plays an important role in processes underlying memory and learning in behaving animals (Altman and Normalie, 1988).

2. Motor control

5-HT is thought to have an important role in motor function (Jacobs, 1991; Jacobs and Fornal, 1993; Jacobs, 1994). It is well established that axons of pyramidal neurons project to regions of the brain such as the basal ganglia, the brainstem and the spinal cord to control motor output (Lorente De No, 1949). Clearly, in the presence of NMDA receptor activation, 5-HT could increase the gain of motor output. However, only a subset of pyramidal neurons was observed to contain 5-HT_{2A} receptor mRNA, so it remains to be established that such control takes place. Activation of 5-HT_{2A} receptors also facilitates glutamate evoked activity of facial motoneurons (McCall and Aghajanian, 1979). Thus, it is interesting to speculate that 5-HT acting at 5-HT_{2A} receptors could play an important role in controlling motor output at multiple levels of the neuroaxis.

3. Cortical arousal

In recent years, a growing body of evidence suggests that atropine resistant neocortical low voltage fast activity is dependent on the ascending 5-HT system in freely moving rats (see section 1.2.10). However, classical 5-HT_{2A} antagonists such as ritanserin, ketanserin, mianserin, pirenperone and methysergide are ineffective in blocking this activity (Vanderwolf, 1988; Watson *et al.*, 1992).

In contrast, in urethane anaesthetized rats, 5-HT_{2A} receptors are responsible for cortical desynchronization induced by noxious stimulation (Neuman and Zebrowska, 1992). A functional interaction between NMDA and 5-HT_{2A} receptors is thought to account for the desynchronization since classical 5-HT_{2A} receptor antagonists such as ritanserin and cinanserin block this activity. These findings are in accord with the present investigation in which similar antagonists significantly reduced the 5-HT_{2A} receptor mediated facilitation of the NMDA depolarization of cortical neurons.

There is an obvious discrepancy between the observations of Vanderwolf and colleagues (Vanderwolf, 1988; Watson *et al.*, 1992) and those of Neuman and Zebrowska (1992). One way to resolve these differences is to consider the involvement of 5-HT_{2A} receptors to be state dependent. Under urethane anaesthesia, which is similar to slow wave sleep, the burst-pause pattern of cortical unit discharges likely results from NMDA receptor activation (Armstrong-James

and Fox, 1986) and the bursting is enhanced by 5-HT_{2A} receptor mediation (see section 1.2.10, Chapter 1). On the other hand, in awake animals, cortical firing might be dependent on AMPA receptors and the cortical desynchronization could be associated with some other excitatory effect of 5-HT. This is in agreement with the evidence that 5-HT fails to facilitate the AMPA component of neocortical EPSP, whereas it does facilitate the NMDA component (Reynolds *et al.*, 1988). Therefore, state dependency of the NMDA component could explain the variation in effectiveness of 5-HT_{2A} receptor antagonists in awake vs anaesthetized animals. Finally, in man, 5-HT_{2A} receptor antagonists have little effect on the waking EEG but they do increase the duration of slow wave sleep (Idzikowski *et al.*, 1986), a finding in keeping with the above interpretation.

5-HT_{2A} receptors in therapeutics

5-HT_{2A} receptors have been postulated as targets for numerous drugs with potential for the treatment of a variety of neuropsychiatric disorders. For example, most antidepressants (tricyclic antidepressants, selective serotonin reuptake inhibitors and monoamine oxidase inhibitors) down-regulate 5-HT_{2A} receptors (Stahl, 1994). Given the findings presented in this thesis on the loss of 5-HT_{2A} receptor function during aging, one wonders whether a similar loss may take place during treatment with antidepressants. This remains to be investigated. In keeping

with this suggestion, ritanserin, a selective 5-HT₂ receptor antagonist has shown promise as an antidepressant (Sleight *et al.*, 1991).

Clinical evidence also suggests that blockade of 5-HT_{2A} receptors by ritanserin and clozapine alleviate the negative symptoms of schizophrenia, an effect which may account for the action of atypical neuroleptics (Siever *et al.*, 1991; Meltzer and Nash, 1991). Moreover, risperidone, a combined 5-HT_{2A} and dopamine receptor antagonist is effective in treating both positive and negative symptoms of schizophrenia (Megnes *et al.*, 1994).

There is abundant experimental data that 5-HT_{2A} receptors play a critical role in the mechanism of action of hallucinogenic drugs such as lysergic acid diethylamide (LSD) and phenylisopropylamine (Glennon *et al.*, 1985; Titeler *et al.*, 1988; Sadozt *et al.*, 1989). There is some controversy as to whether these actions can be explained by agonist or antagonist actions (Pierce and Peroutka, 1988; Glenon, 1990), however, the balance of evidence suggests that most classical hallucinogens, such as LSD, act as agonists at 5-HT_{2A} receptors (Leonard, 1994). A secondary role of 5-HT_{2C} receptors in this mechanism has also been suggested (Titeler *et al.*, 1988; Glennon, 1990).

Some outstanding issues with respect to 5-HT_{2A} receptor activity

5-HT_{2A} receptors exhibit some unusual properties which remain to be explained.

1. 5-HT_{2A} receptors in physiological vs pathological conditions

Leysen (1990) has proposed that 5-HT_{2A} receptors receive low levels of stimulation during normal physiological conditions and only really function in emergency or pathological conditions. Evidence supporting this hypothesis comes from behavioral data following administration of 5-HT_{2A} receptor agonists and antagonists along with the down-regulation of 5-HT_{2A} receptors observed with both agonists and antagonists (see Leysen, 1990; Leysen and Pauwels, 1990 for review). Certainly the results presented in this thesis make it clear that 5-HT_{2A} receptors exhibit multiple and overlapping forms of desensitization, particularly when compared to the mGluR responsible for the NMDA facilitation that exhibits only heterologous desensitization. Several findings are inconsistent with Leysen's hypothesis, e. g. acute administration of 5-HT_{2A} receptor antagonists block cortical desynchronization *in vivo* (Neuman and Zebrowska, 1992) and ritanserin increases slow wave sleep in humans (Idzikowski *et al.*, 1986; Sharpley *et al.*, 1990) suggesting that 5-HT_{2A} receptors are indeed active in physiological conditions such as sleep. Moreover, as revealed previously (see Chapter 6) lack of response to 5-

HT_{2A} receptor stimulation could simply reflect age related changes in the animals used.

2. Atypical down-regulation of 5-HT_{2A} receptors

Chronic administration of 5-HT_{2A} antagonists and, in some cases, a single injection, leads to down-regulation and behavioral desensitization instead of up-regulation and behavioral supersensitivity of 5-HT_{2A} receptors (Conn and Sanders-Bush, 1987a; Leysen and Pauwels, 1990). The down-regulation reflects loss of a functional response which cannot be simply accounted for by a down-regulation in 5-HT_{2A} binding sites. Atypical down-regulation has been suggested to arise as a result of constitutive activity of 5-HT_{2A/2C} receptors (agonist independent PI hydrolysis) and novel properties of 5-HT_{2A/2C} receptor antagonists (Barker *et al.*, 1994; Sanders-Bush, 1994). Sanders-Bush (1994) has suggested that 5-HT_{2A/2C} receptor antagonists are of two types: 1) inverse agonists that block basal constitutive activity of the receptor (i.e PI hydrolysis) as well as blocking 5-HT effects; 2) neutral antagonists that block only 5-HT effects. The inverse agonists were found to initiate down-regulation suggesting a novel signal transduction pathway involved with these receptors (Barker *et al.*, 1994; Sanders-Bush, 1994). Although the findings of Sanders-Bush and colleagues may account for atypical down-regulation, the constitutive activity of 5-HT_{2A} receptors does not appear to

be of functional importance. Thus, there was no evidence that 5-HT_{2A} receptor antagonists decreased the NMDA response in the absence of exogenous agonists (see Chapter 2). A decrease would be expected if constitutive activity contributed to the facilitation, although a decrease in the NMDA response, had it occurred, could have resulted from either blocking the action of released 5-HT at 5-HT_{2A} receptors or constitutive activity.

Future directions

The present investigation has raised several issues which should be addressed in future studies. They are as follows:

1. Despite the usefulness of cortical wedges in investigating 5-HT function, they have a number of limitations which include i) the development of long lasting desensitization, and ii) possible indirect effects. To further investigate the role of 5-HT, a fresh start should be made by employing whole cell recording from acutely dissociated cortical neurons. This should minimize possible indirect actions, allow visualization of the cells and reduce desensitization of 5-HT_{2A} receptors by allowing brief application of agonists.
2. From the present investigation, it is concluded that the IP₃-Ca⁺⁺ pathway

underlies the NMDA facilitation. However, with the methods employed in the present investigation, it was impossible to evaluate the role of IP_3 . Thus, to establish a coherent picture of 5-HT facilitation, the role of the IP_3 receptor in regulating the NMDA current should be examined using whole cell recording. To accomplish this, caged IP_3 released with ultraviolet irradiation could be used.

3. How a rise in intracellular Ca^{++} is translated into facilitation of the NMDA response remains to be answered. Manipulation of intracellular Ca^{++} by various Ca^{++} mobilizing agents should be examined in dissociated neurons. Furthermore, kinetics of the expected rise in intracellular Ca^{++} during the NMDA facilitation needs to be addressed.

4. Presuming that phosphorylation of the NMDA receptor or nearby site is responsible for the facilitation, does such a phosphorylation in turn influence divalent cation and polyamine sites on the NMDA receptor? Whether these sites have an impact on the NMDA facilitation remains to be answered.

5. In senescent rats, activation of protein kinase C is responsible for loss of the facilitation. When and how this process develops is worthy of further examination. Are there losses in cognitive function which parallel loss of the $5-HT_{2A}$ receptor

function? An investigation to examine this hypothesis would facilitate a clearer understanding of 5-HT and its role in cognitive function.

6. As evident in this thesis, 5-HT_{2A} receptors and 5-HT_{1A} receptors produce opposite effects on the NMDA depolarization. Both receptors are located on the same neurons (see Chapter 1) and yet the 5-HT_{2A} receptor effect dominates. This suggests there may be a significant functional interaction between these receptors. This issue should be addressed.

Answers to these questions may contribute to a better understanding of the physiology and pharmacology of serotonergic transmission and functional transmitter interactions in the neocortex. Most importantly, understanding actions and interactions of these neurotransmitters may facilitate the elucidation of potential targets for novel drug therapies for neuropsychiatric disorders and age related cognitive deficits.

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